OVEREXPRESSION OF WUSCHEL IMPROVES THE INDUCTION OF EMBRYOGENIC CALLUS IN SCALPS OF *MUSA ACUMINATA* L. AAA, CV. "GRAND NAIN" †

[LA SOBREEXPRESIÓN DE WUSCHEL MEJORA LA INDUCCIÓN DEL CALLO EMBRIOGÉNICO EN BROTES MERISTEMÁTICOS DE MUSA ACUMINATA L. AAA, CV. "GRAND NAIN"]

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SUMMARY

Background: During the last few years the home box transcription factor *WUSCHEL* (WUS) has been shown to cause dedifferentiation when expressed on somatic cells followed by a production of new stem cells that can lead to somatic embryogenesis or organogenesis. WUS has been shown to promote the transition from a vegetative to an embryogenic state when overexpressed. **Objective/Hypothesis**. The genetic *in vitro* transformation of meristematic tissue of *Musa acuminata* L. AAA, cv. "Grand Nain" was carried out using a heterologous gene *WUSCHEL* from *Arabidopsis thaliana*, via vacuum infiltration with *Agrobacterium tumefaciens*, to establish and ascertain if its expression modifies the progression of the explants to the embryogenesis process and or reduces the time needed for *in vitro* embryogenic induction phase. **Methodology**: Explants of proliferating shoot meristems, named "scalps", of *Musa acuminata* L. AAA, cv. "Grand Nain" was transformed with WUS gene under the control of promoter GAL4, inducible by 17β-estradiol. The effect of steroid 17β-estradiol (β–Est) and homobrassinolide (HomoBra) on the *in vitro* somatic embryogenesis induction phase of non-transformed banana scalps was investigated. **Results**: The successful transformation of the explants was confirmed by PCR, for the transferred neomycin phosphotransferase II (*NPT*II) and the WUS gene. In addition, the expression of the red fluorescent protein (RFP) for the corresponding transferred reporter gene was verified by fluorescence microscopy in proliferated transformed tissues. Besides, the transformed tissue response to the induction of embryogenesis with either β–Est and/or HomoBra, inducers of the transgene were investigated. **Implications:** The improvement of the process of somatic embryogenesis in this way,

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generates a more effective and productive study model in a short time. **Conclusions:** WUS can promote the meristematic tissue-to-embryonic transition, and eventually somatic embryo formation, suggesting that the homeodomain protein can play a critical role during embryogenesis.
Keywords: *A. tumefaciens*; *Musa acuminata*; Estradiol derivatives; Genetic transformation; Homeobox; Somatic embryogenesis; WUSCHEL.

RESUMEN

Antecedentes: Durante los últimos años, se ha demostrado que el factor de transcripción *WUSCHEL* (WUS) causa desdiferenciación cuando se expresa en células somáticas, seguido de una producción de nuevas células madre que pueden conducir a la embriogénesis somática u organogénesis. Se ha demostrado que WUS promueve la transición del estado vegetativo al embriogénico cuando se sobre expresa. **Objetivo/Hipótesis**. Llevar a cabo la transformación genética *in vitro* del tejido meristemático de *Musa acuminata* L. AAA, cv. "Grand Nain" utilizando un gen heterólogo *WUSCHEL* de *Arabidopsis thaliana*, mediante infiltración al vacío con *Agrobacterium tumefaciens*, para establecer y determinar si su expresión modifica la progresión de los explantes al proceso de embriogénesis y/o reduce el tiempo necesario para la fase de inducción embriogénica *in vitro*. **Metodología**: Explantes de meristemos de brotes proliferantes, denominados meristemos "desnudos", de *Musa acuminata* L. AAA, cv. "Grand Nain" se transformó con el gen WUS bajo el control del promotor GAL4, inducible por 17β-estradiol. Se investigó el efecto del esteroide 17β-estradiol (β–Est) y homobrassinolide (HomoBra) en la fase de inducción de embriogénesis somática *in vitro* meristemos desnudos de banano no transformado. **Resultados**: La transformación exitosa de los explantes fue confirmada por PCR, para la transferencia de neomicina fosfotransferasa II (*NPT*II) y el gen WUS. Además, la expresión de la proteína fluorescente roja (RFP) para el gen reportero transferido correspondiente, se verificó mediante microscopía de fluorescencia en tejidos transformados proliferados. Igualmente, se investigó la respuesta tisular transformada a la inducción de embriogénesis con β–Est y/o HomoBra, inductores del transgen. **Implicaciones**: La mejora del proceso de embriogénesis somática de esta manera, genera un modelo de estudio más efectivo y productivo en poco tiempo. **Conclusiones**: WUS es capaz de promover la transición meristemática de tejido a embrión, y eventualmente la formación de embriones somáticos, lo que sugiere que la proteína homeodominio puede desempeñar un papel crítico durante la embriogénesis.
Palabras clave: *A. tumefaciens*; *Musa acuminata;* Derivados del estradiol; Transformación genética; Homeobox; Embriogénesis somática; WUSCHEL.

INTRODUCTION

The banana is a fruit that is grown in all tropical regions of the world and is very important for the social and economic development of local economies in many developing nations. Bananas are a staple meal around the world and are one of the most significant food crops, making them a product for export (Galarza-Suárez, 2019). However, its productivity yield is frequently diminished by various biotic or abiotic adversities. Regardless of the existence of more than 1,000 different varieties, most domesticated types are highly sterile polyploids. Sterility has historically hindered conventional breeding programs, as it has similarly affected plant propagation and germplasm management.

To date, there are different *in vitro* propagation systems in bananas that allow the production of large quantities of plant material with defined genetic characteristics. Various methods have been initially documented for the induction of somatic embryos in bananas, which vary depending on the specific type of explant used. These explants include zygotic embryos (Cronauer-Mitra and Krikorian, 1988), foliar bases and corm slices (Novak *et al.*, 1989), meristems cultured in vitro (Dhed'a *et al.*, 1991), immature males (Escalant *et al.*, 1994), and female flowers (Grapin *et al.*, 2000; Pérez-Hernández and Rosell-García, 2008). Despite the many options, the most widely applicable methodologies for the establishment of embryogenic cell suspensions and regeneration of plants from valuable seedless cultivars have been restricted to the use of either *in vitro* proliferating meristems (Xu *et al.*, 2005; Strosse *et al.*, 2006; Sipen and Davey 2012) or immature flowers as starting material (Côte *et al.*, 1996; Ganapathi *et al.*, 1999; Youssef *et al.*, 2010; Nandhkumar *et al.*, 2018). *Musa acuminata* L. AAA, cv. "Grand Nain" is a recalcitrant cultivar with a somatic embryogenesis (SE) response that fluctuate around 5.4-10.4%, while other related cultivars are below 1% under optimal conditions of development (Youssef *et al.*, 2010). Nevertheless, in banana is still far from being considered a routine technology and has not even been successfully applied to some cultivars (Schoofs *et al.*, 1999; Pérez-Hernández and Rosell-García, 2008; Jafari *et al.*, 2015).

To improve the embryogenic response of banana tissues and increase the production of high-quality suspensions of embryogenic cells, it is imperative to employ biotechnological approaches that integrate *in vitro* culture methodologies with genetic engineering techniques. The activation of numerous genes has been observed to be linked to changes in biochemical and physiological processes across the developmental pathway, with certain genes directly implicated in morphogenesis and development (Karami *et al.,* 2009). Several types of genes, including SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK), BABY BOOM (BBM), LEAFY COTYLEDON (LEC), and WUSCHEL (WUS), have been identified as molecular markers that indicate the occurrence of these alterations across various stages of development (Yang and Zhang, 2010; Neelakandan and Wang, 2012).

One possible solution is the introduction of heterologous genes that stimulate the induction of specific genes that result in the formation of somatic embryos. In A. thaliana, over-expression of genes such as the WUS and BBM have been shown to be involved in the regulation of embryogenesis from the tissue of different explants (Laux *et al.,* 1996). The WUS gene is one of the genes that have aroused considerable interest. In *A. thaliana* mutant analysis suggest that this gene encoding a transcription factor of the homeobox family is required to maintain identity and functionality of the cells at the center of the apical meristem, as well as floral meristem (Mayer *et al.,* 1998; Dolzblasz *et al.,* 2016; Jha *et al.,* 2020). Another gene would be the BBM gene which codes for a transcription factor of the family AP2/ERF. The members of this family are involved in the control of events as the determination of the identity of the floral organs that are expressed preferentially in the development of embryos and seeds, and the formation of somatic embryos in *A. thaliana* by stimulating their overexpression (Boutilier et. al., 2002; El Ouakfaoui *et al* 2010; Chen *et al.,* 2022). Genetic analysis indicates that WUS protein interacts with many other regulators of meristematic outbreaks and floral meristems suggesting that WUS integrates positional and temporal information of these genes as is the case of CLAVATA (CLV) genes and SHOOT MERISTEM LESS (STM) genes (Klaus et. al., 1998). Experimental data suggest that gene regulatory networks of development in plants appear to have been preserved during evolution and different reports indicating that probably the same gene pool is involved in the control of both zygotic embryogenesis and somatic embryogenesis (Dodeman et. al., 1997; Winkelmann T. 2016).

In the past, in vitro experiments with different species of transformed plants via *A. tumefaciens* were done. The heterologous gene WUS was chosen to improve the recalcitrant quality of this species. Previous overexpression in *A. thaliana*, *Coffea canephora*, and *Capsicum chinense* promoted the vegetative-to-embryogenic transition and eventually led to somatic embryo formation (Zuo *et al.*, 2002; Arroyo-Herrera *et al.*, 2008; Solis-Ramos *et al.*, 2009).

Plant steroids have also demonstrated effects on plant growth and development (Fridman and Salvaldi-Goldstein, 2013; Nolan *et al.,* 2020). Physiological, biochemical, and molecular genetic studies have shown that brassinosteroids (BRs) are essential for cell expansion, skotomorphogenesis, apical dominance, leaf and chloroplast senescence, male fertility, and gene expression. Zuo *et al.* (2000a) demonstrated that the estrogen 17-β-estradiol induces target gene expression without causing growth defects in *Arabidopsis*. The estrogen-responsive DNA activator system (XVE) consists of the bacterial repressor LexA, herpes simplex virus protein 16, and the human estrogen receptor. The XVE system has been applied to other plants, such as tobacco BY2 cells (Zuo *et al.*, 2002), tomato (Zhang *et al.,* 2006), *Oryza sativa* (Okuzaki *et al.,* 2011), and *A. thaliana* (Weiste *et al.,* 2017). Also, the chemical-inducible XVE system is a useful tool for determining the function of genes that cannot be examined by their overexpression in plants, such as genes that affect morphology or cause growth defects (Solis-Ramos *et al.,* 2009; Klimaszewska *et al.,* 2010). However, limited information is available regarding the efficacy of estradiol treatment and the level of target gene expression induced by the XVE system in *Musa acuminata* L. AAA, cv. “Grand Nain”; especially in this recalcitrant plant to *in vitro* morphogenesis (Jafari *et al.,* 2015). This paper describes the efficacy of estradiol treatment and the genetic transformation of proliferating meristematic tissue of *Musa acuminata* L. AAA, cv. “Grand Nain” called “scalps” carried out via *A. tumefaciens*. Based on our results, we observed that the transformed scalps with the WUS gene increased their capacity for somatic embryo formation and ectopic morphogenesis.

MATERIALS AND METHODS

Plant material and Culture conditions

Proliferating shoot meristems called “scalps” (Dhed´A et. al., 1991; Shoofs, 1997; Shipen and Davey 2012 Strosse et. al., 2003) of *Musa acuminata* L. AAA, cv. “Grand Nain” (EG) were

used containing 2-4 meristematic domes. Scalps were cultured according to the methodology described by Shoofs (1997), using the medium P4 medium composed of MS (Murashige and Skoog, 1962), supplemented with sucrose (30 g^{-L}), Ascorbic acid (10 mg $^{-L}$), Acid naphthalene acetic (AIA, 0.175 mg $^{-L}$), Benzyl amino purine (BAP, 22.5 µg $^{-L}$) and solidified with gelrite (2 g $^{-L}$) (Shoofs, 1997; Strosse et. al., 2003). The explants were then transferred to the culture room in complete sterility at 27 ± 2°C, 12 h photoperiod (45 µmol m^{-2} s^{-1}) for 30 days. These scalps were used for the experiments of transformation. In addition, somatic embryogenesis was induced using scalps inoculated on ZZ callus induction medium. This medium is of the same composition of P4 medium (MS, salts and vitamins) except that MS macronutrients were reduced to one half and the growth regulators were replaced with trans-zeatin-riboside (1µM) and 2,4-dichlorophenoxyacetic acid, 2,4-D (4.99 µM), (Strosse et. al., 2003).

Plasmid binary constructs

The binary vector generated, pER10W:35SRed, contains the DsRFP reporter gene under the 35S constitutive and the gene WUS under an estradiol-inducible transcriptional control (Zuo *et al.,* 2002). The plasmid pER8 provides expression of GFP (Zuo *et al.,* 2002). The vector was derived through the process of subcloning the gene DsRFP from the plasmid pRSET BRED, which harbors the DsRFP gene within the EcoRI-BamHI restriction sites. This plasmid was generously provided by Meredith Gould from Ens Baja California. The DsRFP gene was removed from pRSET BRED by digesting the plasmid with XbaI, followed by a fill reaction with Klenow fragment and subsequent HindIII restriction digestion. The plasmid pCD includes a 35S promoter sequence, as reported by Gallie *et al*. in 1989. The plasmid underwent digestion using the SalI restriction enzyme, followed by a fill reaction employing the Klenow fragment, and was then treated with the HindIII restriction enzyme. The DsRed-containing DNA was ligated into the digested pCD plasmid with T4 ligase for 8 h at 15 oC. By digesting SmaI and SacI from the new vector called pCD-35SRed, the 35S-DsRFP DNA fragment was made. These DNAs were subcloned into pER10 W donated by Chua NH (Rochefeller University) by digesting pER10 W with SpeI. The ligation reaction was carried out after filling in the 5´overhangs with DNA polymerase I (Klenow fragment, Invitrogene Life Technologies).

Effect of the 17β-estradiol (β-Est) and homobrassinolide steroids on transformed scalps growning on P4 medium, and embryogenesis induction ZZ mediun of banana AAA, cv. "Grand Nain"

Banana scalps were cultured in P4 basal medium, or ZZ medium added with the respective sterol. Three concentrations of β-Est, 0.05, 0.5 and 5.0 µM; and two concentrations of HomoBra, 0.05 and 0.5 µM were tested. Both sterols were dissolved in absolute ethanol (EtOH), the final concentration of the solvent in the medium being 0.1% (v/v). Independent experiments were performed with two replicates (n=4 explants/replicate) each using two culture systems. The embryogenesis induction system in semisolid ZZ medium and the proliferation of scalps in semi-solid P4 medium are described by Strosse *et al*. (2003). These media were added with 5 µM of βEst + 0.05% DMSO and 0.05 µM HomoBra + 0.05% EtOH, respectively. The tissue was cultured for three months and kept under light photoperiod conditions (60 µmol m^{-2} s^{-1} PPFD) 16 h light and 8 h darkness at 27 ± 2 °C. Explants of the different treatments were evaluated every 15 days during the three months. For that purpose, changes in tissue appearance, increase in explant size, degree of differentiation and/or dedifferentiation, formation of different types of callus in ZZ medium (I, II, III and IV, according to the criterion of Strosse *et al*, 2003) and degree of oxidation were recorded. The observations were made with the naked eye and with the aid of a Wild-Photomacroskop M400- (Leitz) stereoscope, with which the photographic records were taken.

Activation of *Agrobacterium* strains and Transformation by vacuum infiltration

The Agrobacterium C58C1 (pER10W-35SRed) cells were cultured in 20 mL of liquid Luria-Bertani (LB) medium (10 g $^{-L}$ tryptone, 5 g $^{-L}$ NaCl, 5 g^{-L} yeast extract) containing the appropriate antibiotics and allowed to grow at 28°C with 200 rpm agitation for two days. Subsequently, 200 mL of the bacterial suspension was added to 10 mL of fresh LB medium supplemented with the required antibiotics, and the culture was allowed to grow at 28°C with 200 rpm agitation for 24 h. Followed by the addition of 10 mL LB medium and 100 mM of acetosyringone into the culture of 24 h. It was incubated for 4 h. The bacterial suspension was pelleted at 5,000 rpm for 5 min at room temperature and resuspended in 20 ml of liquid MS medium containing the growth regulators (3R), and 200 mM acetosyringone until it reached 0.2 OD 600 nm.

The transformation of meristematic tissue of banana plants was done as previously described (Acereto Escoffié et. al., 2005). Briefly, the scalps transferred to the bacterial culture (grown in YEP medium) were vacuum infiltrated at 400 mmHg for 4 min. Then explants were transferred to MS medium with 200 mg $^{-L}$ timentin (Smith Kline Beecham) and incubated in darkness at 27 °C with agitation (110 rpm) for 24 h. Subsequently, the tissues were treated with 200 mg $^{-L}$ timentin and claforan (Aventis) to eliminate the remaining bacteria. Controls were given the same treatment but lacked the addition of *A. tumefaciens*.

Corroboration of WUSCHEL gene overexpression on meristematic tissue of "Grand Nain" cultivar

Genomic DNA was isolated from meristematic tissue of non-transformed and transformed of *M. acuminate* L. AAA, cv. "Gran Nain" from *in vitro* plantlets of 15 or 90 days of development. Meristematic tissues were macerated in liquid nitrogen, with 1 mL of extraction buffer followed by Phenol-chloroform extraction the final pellet after ethanol precipitation was resuspended in 20 mL distilled nuclease free water (Acereto Escoffié *et al.*, 2005). The samples were stored at -20ºC. The extraction of DNA from C58C1 *A. tumefaciens* strain the T-DNA (from the pER10 W-35SRed) was carried out according to the manual for plasmid purification (Wizard PLUS, PROMEGA). The primers for PCR amplification used correspond to the WUS sequence forward (5´-ACATATGGAGCCGCCACAG-3´), and reverse (5´-ATCGCCTCCACATTCTTCTT-3´), which amplify an 862 bp fragment. The final composition of the reaction mixture was: buffer 1X, 1.5 mM $MgCl_2$, 0.8 mM dNTPs mix, 0.2 µM forward and reverse primers, 200 ng of sample DNA and 0.04 U/µL Taq polymerase. The reaction conditions were: 95ºC for 2 min, 30 amplification cycles (95ºC for 1 min, 52ºC for 1 min, 72ºC for 1 min) and a final extension step of 10 min at 72ºC. The virE2 selected primers for detection of *A. tumefaciens* were: forward (5´-TGCCCACCAAGGCGGAATT-3´) and reverse (5´-CTTTGCCGACCCATCGA-3´), which amplify an 895 bp fragment. The final composition of reaction mixture was: buffer 1X, 1.5 mM $MgCl_2$, 0.8 mM dNTPs mix, 0.2 µM forward and reverse primers, 200 ng DNA plant sample and 0.04 U/µL Taq polymerase. The reaction conditions were: 94ºC for 30 s, 30 amplification cycles (94ºC for 1 min, 55ºC for 1 min, and 72ºC for 1 min) and a final extension step of 10 min at 72ºC.

Histological analysis and microscopy

The scalp selected tissue fragments were fixed in FAA solution (formaldehyde – acetic acid – ethanol), at 4°C overnight. Afterwards, tissue was dehydrated in ethanol series (50%, 70%, 96%, 100%, 100% and 100%) and embedded with JB-4 embedding resin kit (Polysciences, Inc.). Serial 10 µm thick sections were cut with a rotary microtome (Microm Internacional GmH), which were transferred to polylysine-coated slides. Side with tissue were dewaxed in a Histolclear solution, and tissue double stained with Periodic Acid-Schiff Reagent and Naphthol Blue Black, slides were observed under an optical microscope (Axioplan, Carl Zeiss).

For detection of RFP (Red fluorescence protein) the tissue was fixed in paraformaldehyde 4% (w/v) in saline phosphate buffer (PBS, Phosphate-buffered-saline) at 4°C on agitation overnight. Dehydrated as above, except that 100% ethanol was replaced by 100% butanol, and the tissue was embedded in Paraplast Plus embedding medium (Sigma-Aldrich). After dewaxed and dehydration, the dry slides were observed using a fluorescence microscope (546/12 excitation filter/ 590 band pass filter). Images were captured with a monochromatic digital camera AxioCam MRm installed on Axioplan microscope (Carl Zeiss).

For detection of DAPI (4 ', 6-diamidino-2-fenilindol) and blue toluidine dye, the somatic embryos were fixed in FAA solution for 72 h in dark conditions at 4ºC. A gradient of sucrose (10%, 20%, 30%) was made as antifreeze in a PB buffer (10 mM sodium phosphate dibasic (Sigma, S3264) and 2 mM potassium phosphate monobasic (Sigma, P5655), pH 7.2 The samples were embedded in a Leica tissue freezing medium (Leica Biosystem) and then frozen at -27ºC. The blocks were sectioned at 10 µm with a cryostat (Leica Biosystem CM1950) The slides with sample tissue were washed three times with sterile distilled water to remove excess Leica tissue freezing medium, then were treated with 10 µL of Vectashield mounting medium and DAPI to stain the nuclei of plant cells (Vector Laboratories, H-1200) and stored in the dark for 1 h at 4ºC. The DAPI staining signal was detected using the excitation wavelength of 405 nm; the emission wavelength was 461 nm. The images were obtained using a confocal laser scanning microscope (Olympus, FV1000 SW) and the FV10 ASW 3.1 viewer software. Blue toluidine staining was used to support the microscopic characterization. For this, embryo tissue sections (20 µm) were stained with a blue toluidine solution at 0.5% for 1 min. Stained sections were mounted on microscope slides with

DPX mounting medium and sealed the edges with nail polish and visualized using a microscope (Axioplan Zeiss, Germany) at a magnification of 10X.

Statistical analyses

All the experiments were conducted in triplicate on biological duplicates. The percentage of callus formation types III and IV of the meristematic tissue (scalps) response in the ZZ medium was transformed logarithmically into [ln (x + 0.5)]. The data was analyzed by ANOVA-F ($P< 0.0001$ and $P< 0.0002$), and means were compared using the Tukey test using the StatSoft STATISTICA version 7.1 package software (www.statsoft.com).

RESULTS

Effect of the 17β-estradiol (β-Est) and homobrassinolide steroids on scalps of banana *Musa acuminata* L. AAA, cv. "Grand Nain" in basal medium (P4)

The effect of β-Est and HBr on tissue of EG banana scalps was evaluated qualitatively considering the degree of phenolization, the growth of the explants and the possible formation of somatic embryos.

After 15 days of initiation of the experiment, it was observed that as the steroid concentration increased, a higher tissue oxidation occurred, as well as a lower scalp proliferation (Fig. 1A and B). The concentration of 5.0 µM β-Est and HBr proved to be toxic to the tissue, since under these conditions the degree of phenolization increased and there was less growth of tissue, compared to the lowest of β-Est, HBr concentrations and the control treatment (Fig. 1A and 1B). Likewise, after 90 days of treatment, it was observed that under the concentration of 0.05 µM β-Est and HBr there was an increased proliferation of white-creamy scalps and differentiation of small areas with green foliar primordia and corm tissue (Fig. 1A and 1B). In addition, a low ethanol (EtOH) concentration was proposed to be added to the treatments for decrease the degree of phenolization. The response of the EG banana scalps to these treatments showed that the concentration of EtOH (0.1%) prevented the phenolization of the explants and even promoted their greater proliferation at 90 days (Fig. 1C).

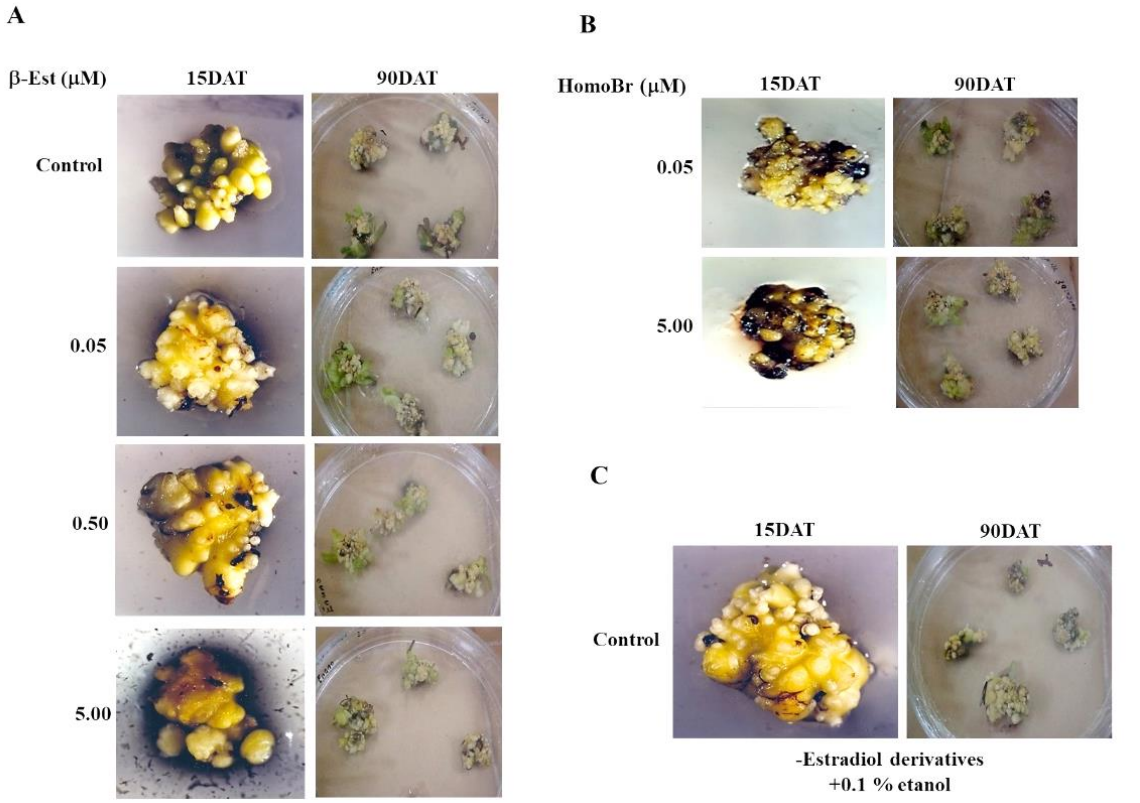

Figure 1. Phenotypic response of scalps of *Musa acuminata* L. AAA cv. "Grand Nain" cultured in presene of different concentrations of 17β-estradiol (β-Est) or 22(S), 23(S) homobrassinolide (HomoBra) in proliferative medium. Scalps were cultured in presence of A) β-Est (0.05, 0.5 and 5.0 µM) or B) HomoBra (0.05 and 0.5 µM) in P4 medium, and phenotypic response recorded 15 days (15DAT) or 90 days after treatment (90DAT). C) As control we used scalps cultured without β-Est or HomoBra in P4 medium.

Effect of the 17β-estradiol (β-Est) and homobrassinolide steroids on formation of embryogenic callus of scalps of banana *Musa acuminata* L. AAA, cv. "Grand Nain" growing on inducer ZZ medium

Although somatic embryogenesis is a standard methodology established in banana, the bottleneck lies in the low efficiency of obtaining ideal callus (IC) available for the initiation of embryogenic cell suspensions (SCE) cultures with good capacity for plant regeneration.

To study the effect of steroids on the formation of embryogenic callus of scalps, β-Est and HomoBra were used in the embryogenesis induction medium (ZZ medium). The first 15 days of treatment the scalp tissue presented a high degree of phenolization and necrosis. This was probably due to stress from mechanical damage to the tissue during the preparation of the explant for inoculation on the ZZ medium, both in the control treatment and in the treatment with the addition of steroids. After 20 days, tissue responded with the formation of callus, being more evident near the tissues that presented phenolized areas (pa), where the type I callus (CI) was observed, consisting of a pale yellow friable callus, whose cells, upon being easily disintegrated, are phenolized rapidly. Likewise, non-embryogenic the type II callus (CII) was developed at 4 weeks of culture, comprising of a yellow nodular callus with a lower degree of phenolization (nc) (Fig 2A). Type III callus (CIII) is formed by spongy creamy-white callus, observed at 6 weeks of culture (Fig 2B). It should be noted that this callus was sometimes accompanied by a non-embryogenic compact nodular white callus which is not appropriate for the initiation of cell suspension culture. At 7 weeks of treatment, a type IV callus (Fig. 2C) called Schoofs (1997) "ideal embryogenic callus (IC)" is observed, this callus being a mixture of callus type III with the presence of translucent globular embryos on clusters of them (Pe) (Fig 2C). Finally, after 2 months of treatment, within the formation of ideal embryogenic callus a cluster of globular stage of embryos (Fig. 2D) is observed.

Additionally, histological analyzes were performed on tissues submitted to the embryogenic induction treatment in the ZZ medium in the presence or absence β-Est and HomoBra. A longitudinal section of the EG scalp is shown in Fig. 2E just before inoculating in the embryogenesis induction medium ZZ (time 0). In this section the tissue of the parenchyma (p), the vascular tissue (vt), the apical meristem (am) and the leaf primordium (lp) are observed. Figures 2F and 2G show the evolution scalps after 3 months of induction on ZZ medium. Fig. 2F, longitudinal sections of embryogenic callus showing globular embryos with formation of protodermis (pd) originating from a series of periclinal divisions, and de novo proliferation of embryogenic callus (dnc) from globular embryo cells and embryogenic cells (ec). Fig. 2G shows a section of embryogenic callus with a globular pro-embryo (pe) surrounded of embryogenic cells and epidermis (e).

The quantitative analysis of the effect of β-Est and HomoBra on scalps on induction medium ZZ embryogenesis, significant differences ($p < 0.001$) were found between β-Est treatments and controls. The highest percentage of embryogenic callus (31.37%) formation was found at 0.05 μM of β-Est. While HomoBra best response was found at a concentration of 0.5 μM, however the effect of this sterol did not exceed the response induced by β-Est, as it was 17.5% (Fig. 2I). The embryo for each stage of development were observed and analyzed. The dissected embryos were either stained with or with DAPI for nuclear stain. As stated in the legend showed to be normal in shape from globular to cotyledonary stage were detected from the induced tissues.

Histological analysis of embryogenic cell shows normal embryogenic development

We took the developed structures and observed the different stages of embryonic tissue (**Figures 3A, B, C,** and **D),** which show well-defined globular structures of around 600 mM, pear-shaped with an average of 800 mM similar in size, but were the early-coleoptile and cotyledonary phases that would range in a slightly larger size. The structures showed cells with normal-size nuclei (**Figures 3E, F, G, H, I, J, K, L, M, O,** and **P**). The globular structures seen in figure 3e show a well-defined form with a similar type of space between cells to form the spherical structure. Under higher magnification, we can compare cell nuclei in the different stages. The transition from globular to the next stage shows some of the cells having differentiation and nuclear diffuse DAPI stain in the central part of the developed structure, indicating that cells are under a high level of transcription expression. This continues in the following stages, with a reduction near the cotyledonary stage. As such, it shows normal, healthy embryonic tissue being produced.

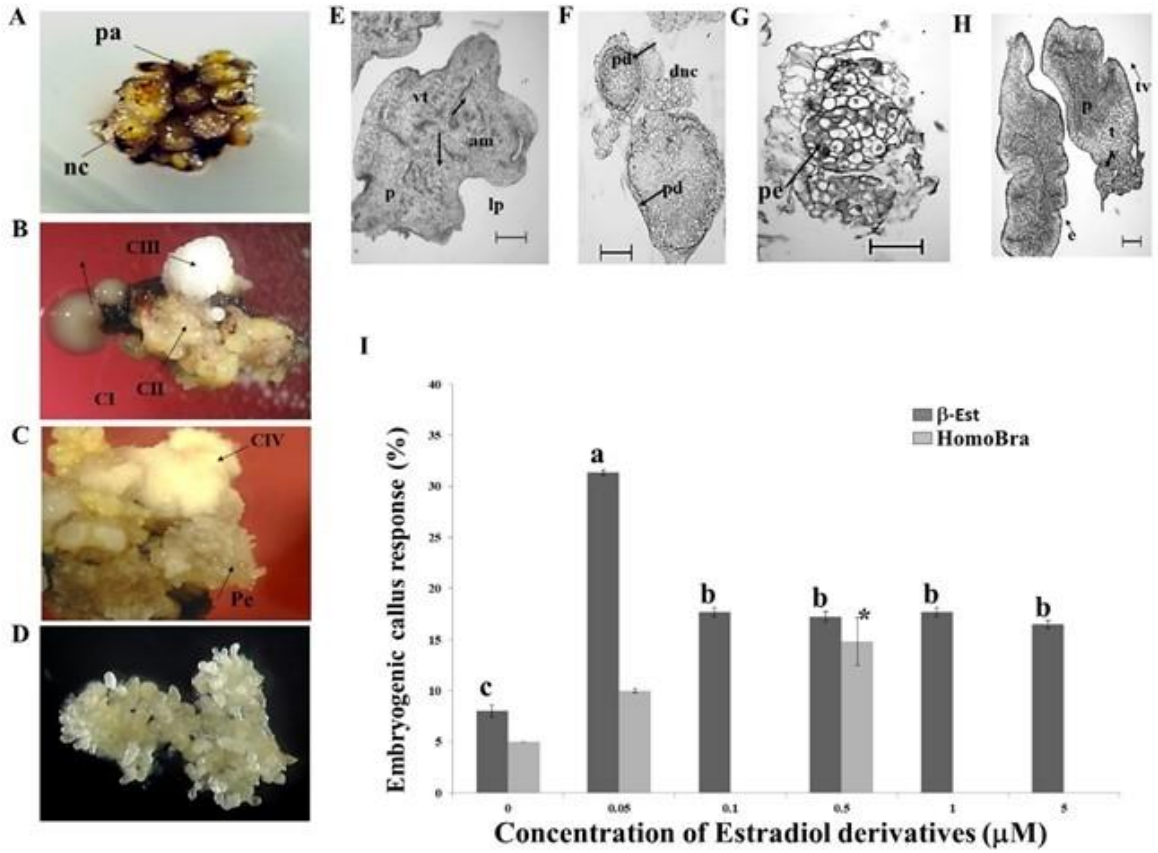

Figure 2. Formation of embryogenic calluses from the scalps of Musa acuminata L. AAA cv. "Grand Nain" ("GN") in ZZ medium for induction of embryogenesis in the presence of steroids A) Scalps after 1.5 months of development present cells of callus-type I (CI) with some phenolized areas (cnf) and non-embryogenic yellow callus-type II (cne). B) Scalps presenting callus-type I (CI), callus-type II (CII), and callus-type III (CIII). C) Scalps presenting callus-type IV (CIV) D) Close-up of clusters of globular embryos from IC (Type IV) at 2 months after the experiment was started. E-G) Histological analysis of callus sections obtained from "GN" scalps on ZZ medium in the presence and absence of β-Est and HomoBra at time zero and H-J) after three months of induction. E) Banana scalp at day zero in induction medium, presenting parenchyma tissue (p), vascular tissue (tv), apical meristem (ma), and foliar primordia (pf). The micrograph scale was taken at 100 mm bar. F) Longitudinal section of an embryogenic callus showing globular embryos with defined protodermis formation (pr) and de novo formation of the callus (cdn). The micrograph scale was taken at 200 mm bar. G) Proembryo (pe) is surrounded by embryogenic cells. The micrograph scale was taken at 100 mm bar. H) Mature somatic embryo with formation of haustorium (h), epidermis (e), and vascular tissues (tv). The micrograph scale was taken at 50 μm bar. I) Effect of sterols, 17 β-estradiol (β-Est) and homobrassinolide (HomoBra), in the scalps of Musa acuminata L. AAA cv. "Grand Nain" in a medium able to induce the embryogenic response. The scalps were cultured in the presence of β-Est (0.05, 0.5, and 5.0 mM) or HomoBra (0.05 and 0.5 mM) in ZZ medium after 90 days after treatment (90DAT). The bars denote the standard deviation of the means of four replicates in two independent experiments. The letters and asterisks denote significant differences at P <0.001.

Genetic transformation of banana meristematic tissue with WUSCHEL gen of *Arabidopsis thaliana* by vacuum infiltration

For the binary vector generated genetic transformation, pER10W:35SRed (Arroyo-Herrera et. al., 2008), contains the DsRFP reporter gene under the 35S constitutive and the WUSCHEL gene under an estradiol inducible transcriptional control (Fig. 4A-a). As expression and fluorescence control, plasmid pER8 is used which contains the green fluorescent protein and is regulated under the inducible 17β-estradiol promoter (Fig. 4A-b). Both plasmids contain the selection gene called *NPT*II which confers resistance to kanamycin under the 35S promoter.

The tissue used for the transformation was meristematic tissue (also named as "scalps") of *Musa acuminata* L. AAA, cv. "Grand Nain" (EG) was used containing 2-4 meristematic domes. The scalps were maintained and subcultured according to the methodology described by Shoofs, (1997), consistent with the basal medium, the scalps were maintained for 30 days at 27ºC ± 2 (Fig 4 B-a). The Agrobacterium C58C1 (pER10W-35SRed) cells were cultured, and the transformation of meristematic tissue of banana plants was done as previously described (Acereto Escoffié *et al.*, 2005) by vacuum infiltration. The Agrobacterium C58C1 was deleted with 200 mg.l^{-L} of thymetin. Once the explants were transformed, the scalps were transferred medium P4 with 40 mg.l^{-L} kanamycin.

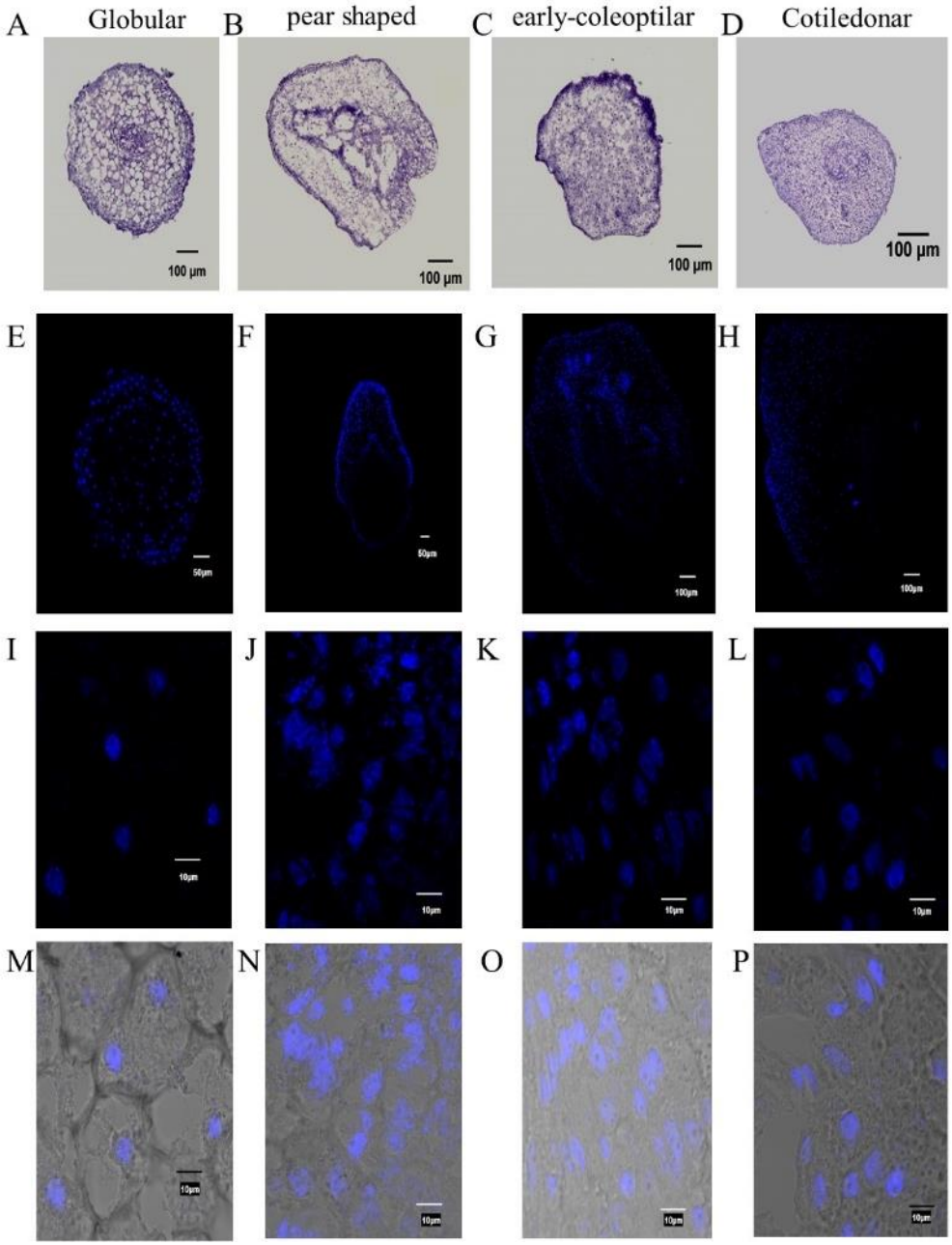

Figure 3. Representative morphological traits on embryo tissue sections at different stages of development Longitudinal cut and blue toluidine staining: A) globular; B) pear-shaped; C) early coleoptylar; D) cotyledon in bright field. Confocal microscopy of the different stages stained with DAPI (E-H). Nuclear stain at low magnification shows the overall structure of each stage (I–L). Higher magnification of the DAPI stain nucleus shows cell differentiation. (M-P) It shows an image merge of phase contrast and DAPI stain cells at the different stages of development.

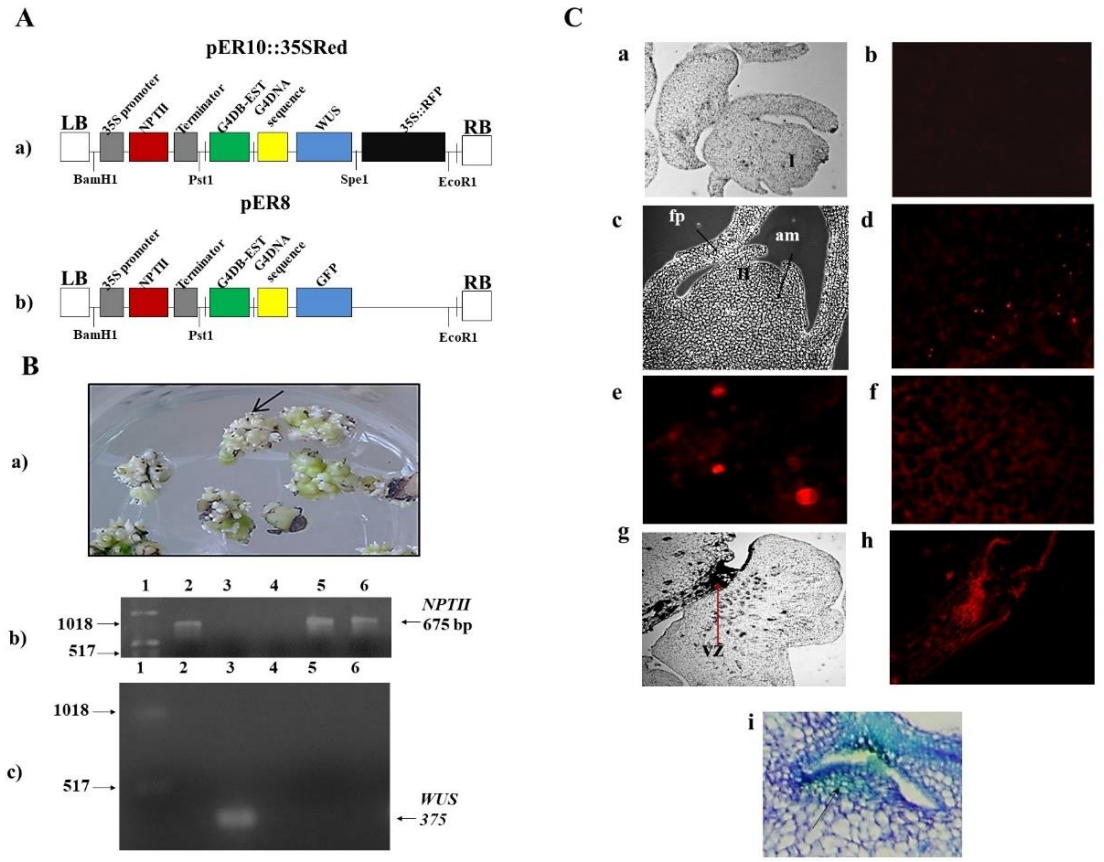

Figure 4. Genetic transformation of scalps of *Musa acuminata* L. AAA, cv. "Grand Nain" (GN) using the WUSCHEL (WUS) gene from *A. thaliana*. A) Schematic representation of the region between the right and left border of the T-DNA of the plasmid: pER10::35SRed (a) or pER8 (b). In green the 17 β-Estradiol inducible promoter which controls expression of the WUS gene (blue box), the red fluorescent protein gene under the operation of the constitutive 35S promoter is shown in black. B) Evaluation of the different scalps of "Grand Nain" transformed with C58C1 *A. tumefaciens* pER10::35SRed. B) Maintenance and propagation of meristematic tissue of banana explants also called "scalps" that were used for the genetic transformation experiments. b) amplification of a fragment of the *NPTI*I gene (genomic DNA) from transformed explants by PCR: Lane 1, DNA ladder (1 Kb); Lane 2, positive control (pCambia 2301 vector); Lane 3, untransformed banana explant; Lane 4, transformed banana explant with the C58C1 *A. tumefaciens* disarmed strain; Lane 5, transformed explant with C58C1 *A. tumefaciens* strain containing the pER8 vector; Lane 6, transformed explants with C58C1 *A. tumefaciens* strain containing the pER10::35SRed construct. c) Amplification of a WUS gene fragment (genomic DNA) of transformed explants by PCR. 1, DNA ladder (1 Kb); 2, negative control (pCambia 2301 vector); 3, transformed explant with C58C1 *A. tumefaciens* strain containing the pER10::35SRed construct; 4, untransformed banana explants; 5, transformed explant with the C58C1disarmed strain; 6: explants transformed with strain with C58C1 *A. tumefaciens* strain containing the pER8 construct. C) Histological sections of segments of transformed scalps with the pER10::35SRed construct and non-transformed of *M. acuminata* L. AAA, cv. "Grand Nain" micropropaged *in vitro*. Sections of non-transformed scalps showed by transmitted light field (a). Amplification of area named delimited with a roman number I showed in the "figure a" showing not presence of the red fluorescence signal from non-transformed meristematic tissue (b). Sections of transformed scalps showed by transmitted light field (c). Sections of transformed scalps by pER10::35SRed construct showing different red fluorescence signals (d, e, f and h). The microphotography showed in the "figure c" showing apical meristem dome and leaf primordia that it was taken under phase contrast. Amplification of area named delimited with a roman number II showed in the "figure c" from a section of scalps showing positive presence of the red fluorescence signal from transformed meristematic tissue (d and e). The microphotography in the "figure g" showed phenolized vascular zones that gives not red fluorescence signal. Transformed meristematic tissue dye by PAS-Naftol blue-black and viewed by transmitted light field (i). Abbreviations: (ma) apical meristem, (pf) leaf primordia, (vz) vascular zones. Plan Objective: 10X and 40X.

The transformation of the scales was verified by the PCR test and histological analysis. For PCR, specific primers were used for the kanamycin resistance gene *NPT*II and the WUSCHEL gene. As a result, amplification of a 675 bp fragment was achieved for the *NPT*II gene (Fig 4B-b) and a 375 bp fragment corresponding to the WUSCHEL gene (Fig 4B-c). Regarding the histological analyzes, fluorescence microscopy was performed to obtain the red fluorescent protein (RFP); the transformed slides transformed with the pER10W-35SRed construct were subjected to fine sections and analyzed by epifluorescence. The analyzed tissues were foliar primordia and meristematic zones. The cuts performed on the untransformed scalps when excited at 546/12 nm, did not show any fluorescence or auto fluorescent regions (Fig. 4C-b). In contrast to the tissues mentioned above, the transformed scalps show different fluorescence signals within the meristematic zone (Figures 4C-d, e, f, and h).

Effect of 17 β-estradiol (β-Est) and homobrassinolide on somatic embryogenesis *in vitro* of transformed meristematic tissue (scalps)

Adding genes that are directly involved in embryogenesis, like the WUSCHEL gene, which helps figure out the identity of the plant's stem cells and apical meristem, seems to be a way to increase the embryogenic response in plants that change their shape, like bananas. We did two separate experiments with two copies of each using the base medium P4 and the ZZ embryogenic induction medium to see how β-Est and HomoBra affected the transformed scalps.

So, the embryogenic response in transformed explants was induced on ZZ medium with 5 M of -Est and 0.05, 0.5 M, and the result was a 6.7-fold higher embryogenic response in the transformed tissues than in explants that hadn't been changed, which was found in the literature for explants that hadn't been changed. However, in basal medium P4, the response was directed towards the path of organogenesis. The embryogenic response of the tissue was analyzed every 15 days for 3 months, following the criteria of Schoofs (1997). The percentage of explants that formed callus types III and IV and the presence of somatic embryos without the formation of an embryogenic callus were estimated (Figures 5A–D).

A 10% embryogenic callus type IV was obtained in the untransformed scalps in ZZ medium. Regarding the transformed explants, embryogenic callus types III and IV were developed, as well as somatic embryos in the control explants observed after 45 days in culture as the transformed explants.

The experimental findings of this study revealed that within the control (without steroids), the presence of the inserts in the tissue modified the embryogenic response. This response increased the formation of Callus III (Table 1, Fig. 5C) from 2.5 to 10 times and from 1.2 to 1.6 times of Callus type IV (Fig. 5D), and the expression of somatic embryos from 1.6 to 2 times. The data showed highly significant differences at the $P < 0001$ and $P < 0.0002$ levels, using the ANOVA-F test.

Induction of individual somatic embryos or small groups without the presence of Callus type IV (Figs. 5A and B) in the transformed explants increased significantly ($P < 0.0001$) in the presence of the steroid and in the absence of kanamycin. It is important to emphasize the difference in the embryo formation response, which fluctuates from 25% in the presence of β-Est to 42% in the presence of HomoBra (Table 1). Moreover, the presence of kanamycin reduces the embryogenic response in transformed tissues by 1.5 to 3 times.

In the other independent experiment, where the transfected scalps were incubated in P4 basal medium with the same concentrations of β-Est and HomoBra, the tissue responses were totally different. After 15 days of initiation of the experiment, an organogenesis response of the cultivated tissue was observed, with an increase in the proliferation of leaf primordia of the corm tissue, as well as bud growth and primary root development (Figures 5F–H). The presence of foliar primordia is observed in a smaller number in the control, corresponding to untransformed tissue (Fig. 5E).

DISCUSSION

Somatic embryogenesis *in vitro* is induced in plant tissues by the action of auxinic-type phytoregulators. Embryogenic callus induction in banana has been achieved with the synthetic auxin 2-4-dichlorophenoxyacetic acid (2, 4-D). However, to date the type IV ideal callus (IC) formation with expression of somatic embryo useful for the induction of embryogenic suspensions, does not exceed 6.2% in bananas of the Cavendish subgroup, which includes the "Grand Nain" cultivar (Strosse *et al.,* 2006). Therefore, it is necessary to examine the role of other phytoregulators with potential to promote better morphogenic responses. Thus, a good candidate would be the use of steroidal derivatives. Brassinosteroids (BRs) are involved in the regulation and signaling of cell division and

expansion, during embryogenic and post-embryogenic development in plants (Jang *et al.*, 2000), during vascular differentiation and in response to stress (Miyazawa et. al., al., 2003). In addition, BRs stimulate the formation of new shoots and promote the regeneration of seedlings from somatic embryos, as in *Cocus nucifera* (Azpeitia et. al., 2003). These compounds, BRs, have structural similarity to steroid hormones of animal origin derived from cholesterol, such as androgens and estrogens, among others (Clouse 2002). Estrogen 17 β-estradiol (β-Est) has been used in various genetic transformation systems as inducer of promoters that are unfold by this compound. For example, it has been used to regulate overexpression of genes introduced in *A. thaliana* (Zuo et. al., 2002). However, it is not known what role this hormone might have in plant cells, if it is perceived by the BRs receptors, and if it also stimulates somatic embryogenesis.

In this study, conversion of type III and IV callus formation (8 % and 4 %, respectively) was observed when we use sterol-type derivatives in an embryogenesis induction medium (ZZ medium) during sixth and eighth week of development from meristematic tissues of "Grand Nain" cultivar. This result is very different from that observed by Strosse *et al*. (2003) in which they reported that the highest frequency of formation of embryogenic callus type IV occurs between the 3 to 6 months before the experiment was carried out, obtaining a percentage of ideal callus (IC) of embryogenic type from 0.1 to 6.2% for different cultivars of bananas from the Cavendish group (AAA), among which the cultivar "Grand Nain" is found from male inflorescence tissue to induce the formation of embryogenic callus. A similar result was obtained for a reproducible regeneration protocol via somatic embryogenesis from plumule from *Coco nucifera* (Azpeitia *et al.*, 2003). These authors found

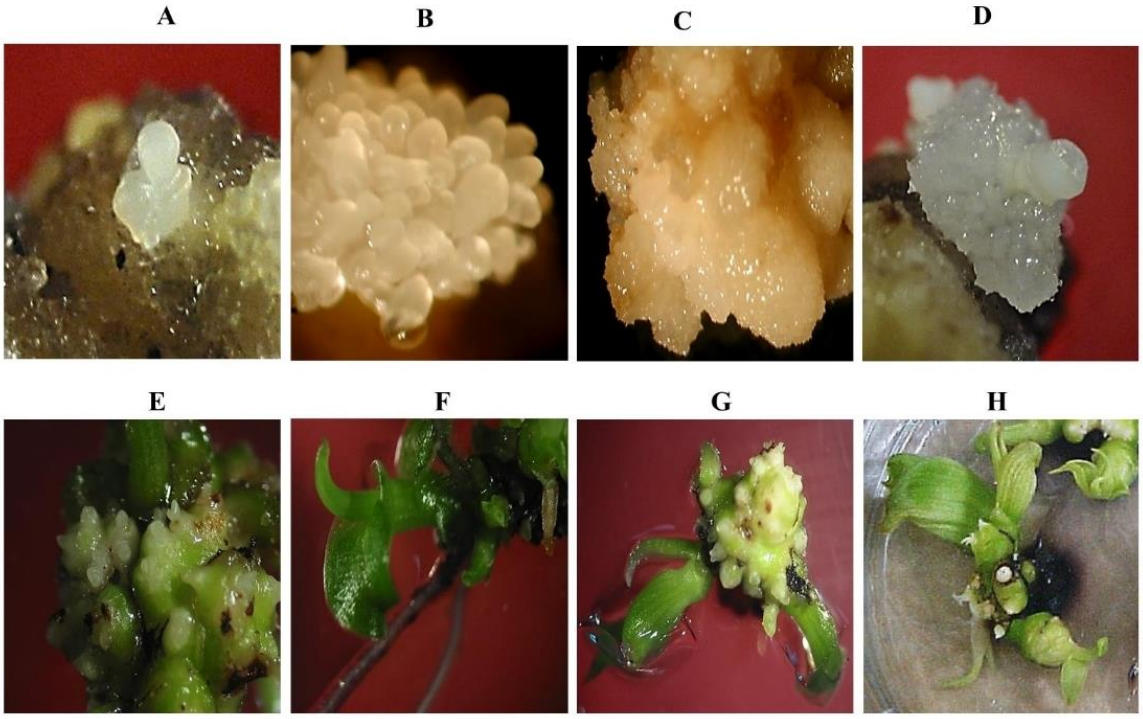

Figure 5. Evaluation of different tissues of *Musa acuminata* L. AAA, cv. "Grand Nain" (GN) transformed with C58C1 *A. tumefaciens* strain containing the pER10::35SRed construct. A) Expression of single somatic embryos; or B) small groups without intermediation of embryogenic callus type III. C) Embryogenic callus type III and D) embryogenic callus type IV, on explants transformed with C58C1- pER10::35SRed construct in ZZ medium in presence with the inductor βEst and/or HBs. E) Development of GN untransformed scalps with proliferating buds, leaf tissue and primary roots. F) Development of GN transformed scalps transformed with C58C1-pER10::35SRed construct and cultured in P4 medium in the presence of βEst. G) Development of GN transformed scalps transformed with C58C1-pER10::35SRed construct and cultured in P4 medium in the presence of HomoBra. H) GN plants of three months old partial regenerated from the meristematic tissue (scalps). The microphotographs were observed with a LEICA stereoscope. Plan Objective: 40X.

Table 1. Embryogenic response of untransformed and transformed GN banana scalps grown in an embryogenesis medium with or without a selection agent (kanamycin) and in the presence of 5 mM β-Est, and 0.05 μM HomoBra, a related plant sterol.

Treatment	Percentage of embryogenic callus formed				Type of Embryogenic callus formed
	Non-transformed plant tissue	C58C1-disarmed strain	C58C1-containing pER8	C58C1-containing pER10FQ	
Control	5 %	0.0 %	13 %	51 %	III
	10%	0.0 %	12.5 %	17.2%	IV
	N.D	0.0 %	26.1 %	16.6 %	Embryos
(+)β-Est	8.4 %	16.9 %	16.6 %	24.0 %	III
(-)Kan	4.2 %	0.0 %	0.0 %	0.0 %	IV
	N.D	0.0 %	8.3 %	26.0 %	Embryos
(+)β-Est	25.2 %	0.0 %	8.1%	13.0 %	III
(+)Kan	16.9 %	0.0 %	N.D	0.0 %	IV
	N.D	0.0 %	8.6 %	0.0 %	Embryos
(+)HomoBra	8.4 %	0.0 %	0.0 %	33.3 %	III
(-)Kan	8.1 %	0.0 %	0.0 %	0.0 %	IV
	N.D	0.0 %	25 %	41.9 %	Embryos
(+)HomoBra	26.0 %	0.0 %	0.0 %	32.0 %	III
(+)Kan	24.0 %	0.0 %	0.0 %	0.0 %	IV
	N.D	0.0 %	8.4 %	23.0 %	Embryos

(+)β-Est: ZZ medium containing 17β-estradiol steroid; (+)HomoBra: ZZ medium containing homobrassinolide steroid; (-)Kan: ZZ medium containing absence of kanamycin; (+)Kan: ZZ medium containing presence of kanamycin; N.D: data not determined.

that the explants responded favorably to the BRs increasing their capacity to form initial callus, embryogenic callus, and somatic embryos. The most significant number of somatic embryos, 10.8 somatic embryos per explant, was obtained when exposing the explants for 3 days to the BR at 0.01 or 0.1 mM, whereas only 3.8 somatic embryos per explant were found from untreated explants (Azpeitia et. al., 2003). On the other hand, Ponsamuel et. al., (1996) reported a protocol of somatic embryogenesis from the immature cotyledonary tissues of cultivated *Camellia sinensis* where the conversion frequency increased a 50% when using a concentration of 1 μM Br.

Chemical-inducible systems have been utilized in the field of genetic engineering for many purposes, including the generation of marker-free transgenic plants (Zuo *et al.*, 2001; Sreekala et. al., 2005; Zhang et. al., 2006; Costa et. al., 2009) and the inducible synthesis of additional proteins (Dohi et. al., 2006; Okuzaki et. al., 2011). These chemical-inducible gene expression systems are powerful tools for controlling the timing and level of target gene expression in plants. Here we report the development a system for the transformation of meristematic tissue of *Musa acuminata* L. AAA, cv. "Grand Nain" using a heterologous gene *WUS* of *A. thaliana* in a chemical-inducible gene expression system.

We selected the hetorologous gene *WUS* of *A. thaliana*, because their overexpression of WUS in *A. thaliana* (Mayer et. al., 1998; Gallois et. al., 2002; Zuo et. al., 2002; Lara Lopes et. al., 2021) and *Coffea canephora* induces embryonic cell clusters (Arroyo-Herrera et. al., 2008), and in *Capsicum chinense* promote the transition from vegetative to embryogenic globular structures state suggesting that heterologous WUS protein was active and involved in the process of ectopic morphogenesis (Solis-Ramos et. al., 2009), and in rice induces multiple shoots (Noriko et. al., 2003). The transformed embryogenic callus was developed from "Grand Nain" cultivar and evaluated by red fluorescence and PCR to determine the presence of the WUS transgene. In all the transformed tissues a fragment of 862 bp corresponding to the expected size for *WUS* gene insert was observed, the fragment did not amplified in the non-transformed tissue of "Grand Nain". Standard PCR for gene virE2 was completed to test the absence of C58C1 *A. tumefaciens*, the results suggest the positive genetic transformation with *WUS* gene during the *in vitro* conversion of different type of embryogenic formation in a medium selected for embryogenic culture (ZZ medium) from scalps of "Grand Nain" cultivar.

After *in vitro* culturing for 45 days, embryogenic callus formation was observed in the transformed meristematic tissue of "Grand Nain", induced with

0.05 mM 17 β-estradiol; from which embryogenic callus type III and embryogenic callus type IV increased their presence (50 % and 16.60 % of response, respectively) in the explant, in the presence of esterols y absence of kanamycin. The literature reports that this embryogenic response occurs between 1 to 5 months (Strosse et. al., 2003), being very different the embryogenic response conversion from the results obtained by us. This data coincides with previous observations from root explants of *A. thaliana* that over-expressed WUS in presence of an inductive medium (17 β-estradiol), producing numerous rapidly growing, yellowish embryogenic calli, that subsequently developed into distinctive somatic embryos (Zuo et. al., 2002); and with other species of plants with the same behavior as in *Coffea canephora* (Arroyo-Herrera et. al., 2008), *Capsicum chinense* (Solis-Ramos et. al., 2009) and *Oryza sativa* (Noriko et. al., 2003).

The genetic transformation of *M. acuminata* L. AAA, cv. “Grand Nain” using meristematic tissue is an essential tool for plant improvement. However, the development of an efficient and reproducible somatic embryogenesis protocol is the first step needed, as a very efficient technology is far from been successfully applicable to different banana cultivars (Pérez-Hernández and Rosell-García, 2008; Jafari et. al., 2015).

CONCLUSIONS

We conclude that WUS can promote the transition from meristematic to embryonic tissue and, ultimately, the formation of somatic embryos, suggesting that the homeodomain protein may play a pivotal role during embryogenesis in addition to its role in meristem development. Furthermore, this regulatory control seems to work well in a heterologous system, such as in M. *acuminata* L. AAA, cv. "Great Nain". Highly restrictive expression of WUS is hypothesized to mark the putative embryonic organizing center, which, in turn, can give rise to stem cells during embryogenesis, followed by a cascade of genes that are involved in development, as mentioned (Shivani *et al.*, 2017). First, exogenously added homobrasinolide does not activate the inducible promoter that controls WUS gene expression, and second, it does not cause an induction of somatic embryogenesis from non-transformed or transformed banana meristems.

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Compliance with ethical standards. Not applicable due to the nature of the study.

Data availability. Not applicable to this article as no datasets were generated or analyzed during the current study.

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REFERENCES

Acereto-Escoffié, P. O. M., Chi-Manzanero, B.H., Echeverría-Echeverría, S., Grijalva, R., Kay A.J., González-Estrada, T., Castaño, E. and Rodríguez-Zapata, L.C., 2005. *Agrobacterium* mediated transformation of *Musa acuminata* cv “Grand Nain” scalps by vacuum infiltration. *Scientia Horticulture*, 105, pp. 359-371. http://doi.org/10.1016/j.scienta.2005.01.028

Arroyo-Herrera, A., Ku-González, A., Canche-Moo, R., Quiroz-Figueroa, F.R., Loyola-Vargas, V., Rodríguez-Zapata, L.C., Burgeff- D’Hondt, C., Suarez-Solís, V.M. and Castaño, E., 2008. Expression of WUSCHEL in *Coffea canephora* causes ectopic morphogenesis. *Plant Cell, Tissue and Organ Culture*, 94(2), pp. 171-180. http://doi.org/10.1007/s11240-008-9401-1

Azpeitia, A., Chan J. L., Saenz, L. and Oropeza, C., 2003. Effect of 22(S),23(S) homobrassinolide on somatic

embryogenesis in plumule explants of *Cocos nucifera* (L.) cultured *in vitro*. *Journal of Horticultural Science and Biotechnology*, 78(5), pp. 591-596. http://doi.org/10.1080/14620316.2003.11511669

Boutilier, K., Offringa, R., Sharma, V.K., Kieft H., Ouellet, T., Zhan,g L., Hattor,i J., Liu, C.M., van Lammeren, A.A., Miki B.L., Custers J.B. and van Lookeren Campagne M.M., 2002. Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. *The Plant Cell*, 14(8), pp. 1737-49. http://doi.org/10.1105/tpc.001941.

Chen, B., Mass, L., Figueiredo, D. and Boutilier K., 2022. BABY BOOM regulates early embryo and endosperm development. *Plant Biology*, 119(25), p. e2201761119. https://doi.org/10.1073/pnas.2201761119

Clouse, S.D., 2002. *Arabidopsis* Mutants Reveal Multiple Roles for Sterols in Plant Development. *The Plant Cell*, 14, pp. 1995-2000. http://doi.org/10.1105/tpc.140930.

Costa, L.D., Vaccari, I., Mandolini, M. and Martinelli, A., 2009. Elaboration of a reliable strategy based on Real-Time PCR to characterize genetically modified plantlets and to evaluate the efficiency of a marker gene removal in grape (*Vitis* spp.). *Journal Agricultural Food Chemistry*, 57, pp. 2668–2677. http://doi.org/10.1021/jf802740m

Côte, F.X., Domergue, R., Monmarson, S., Schwendiman, J., Teisson, C. and Escalant, J.V., 1996. Embryogenic cell suspensions from the male flower of *Musa* AAA cv. Grand nain. *Physiologia Plantarum*, 97, pp. 285-290. http://doi.org/10.1034/j.1399-3054.1996.970211.x

Cronauer-Mitra, S.S. and Krikorian, A.D., 1988. Plant regeneration via somatic embryogenesis in the seeded diploid banana *Musa ornata* Roxb. *Plant Cell Reports*, 7, pp. 23-25. http://doi.org/10.1007/BF00272970

Dhed'a, D., F. Dumortier, B., Panis, D., Vuylsteke and De Langhe, E.A.L., 1991. Plant regeneration in cell suspension cultures of the cooking banana cv "Bluggoe" (*Musa* spp. ABB group). *Fruits*, 46, pp. 125-135. https://www.pubhort.org/fruits/

Dodeman, V. L., Ducreux, G. and Kreis M., 1997. Review article: Zygotic embryogenesis *versus* somatic embryogenesis, *Journal of Experimental Botany*, 48(8), pp. 1493-1509, http://doi.org/10.1093/jxb/48.8.1493.

Dohi, K., Nishikiori, M., Tamai A., Ishikawa, M., Meshi, T. and Mori, M., 2006. Inducible virus-mediated expression of a foreign protein in suspension-cultured plant cells. *Archives of Virology*, 151, pp. 1075–1084. http://doi.org/10.1007/s00705-005-0705-8

Dolzblasz, A., Nardmann, J., Clerici, E., Causier, B., Graff, E., Chen, J., Davies, B., Werr, W., Laux, T., 2016. Stem cell regulation by *Arabidopsis* WOX genes. *Molecular Plant*, 9(7), pp. 1028-1039. http://doi.org/10.1016/j.molp.2016.04.007

El Ouakfaoui, S., Schenell, J., Abdeen, A., Colville, A., Labbé, H., Han, S., Baum, B., Laberge, S., and Miki, B., 2010. Control of somatic embryogenesis and embryo development by AP2 trancription factors. *Plant Molecular Biology*, 74, pp. 313-326. http://doi.org/10.1007/s11103-010-9674-8

Escalant, J.V., Teisson, C. and Côte, F., 1994. Amplified somatic embryogenesis from male flowers of triploid banana and plantain cultivars (*Musa spp.*). *In vitro Plant Cellular and Development Biology*, 30, pp. 181-186. http://doi.org/10.1007/BF02823029

Fridman, Y. and Salvadi-Goldstein S., 2013. Brassinosteroids. In growth control: How, when and where. *Plant Science*, 209, pp, 23-31. http://doi.org/10.1016/j.plantsci.2013.04.002.

Galarza-Suárez, L., 2019. Tierra, trabajo y tóxicos: sobre la producción de un territorio bananero en la costa sur del Ecuador. *Estudios atacameños*, (63), pp. 341-364. http://dx.doi.org/10.22199/issn.0718-1043-2019-0034

Gallie, D.R., Lucas, W.J. and Walbot, V., 1989. Visualizing mRNA expression in plant protoplasts: factors influencing efficient mRNA uptake and translation. *The Plant Cell*, 1, pp. 303–311. http://doi.org/10.1105/tpc.1.3.301

Gallois, J.L., Woodward, C., Reddy, G.V. and Sablowski, R., 2002. Combined SHOOT MERISTEMLESS and WUSCHEL trigger ectopic organogenesis in *Arabidopsis*. *Development*, 129(13), pp. 3207-17. http://doi.org/10.1242/dev.129.13.3207.

Ganapathi T.R., Suprasanna, P., Bapat, V.A., Kulkarni V.M. and Rao, P.S., 1999. Somatic embryogenesis and plant regeneration from male flower buds in banana. *Current Science*, 76, pp. 1228–1231. https://www.jstor.org/stable/24101947

Grapin, A., Ortíz, J.L., Lesco, T., Ferrière , N. and Côte, F.X., 2000. Recovery and regeneration of embryogenic cultures from female flowers of False Horn Plantain. *Plant Cell Tissue Organ Culture*, 61, pp. 237–244. http://doi.org/10.1023/A:1006423304033

Jafari, N., Othman, R. Yasmin, Tan, B. Chin, and Khalid, N., 2015. Morphohistological and molecular profiles during the developmental stages of somatic embryogenesis of Musa acuminata cv. 'Berangan' (AAA). *Acta physiologiae plantarum*, *37*(3), pp. 1796-1796. http://doi.org/10.1007/s11738-015-1796-9

Jang, J.C., Fujioka, S., Tasaka, M., Seto, H., Takatsuto, S., Ishii, A., Aida, M., Yoshida, S. and Sheen. J., 2000. A critical role of sterols in embryonic patterning and meristem programming revealed by the *fackel* mutants of *Arabidopsis thaliana*. *Genes & Development*, 14(12), pp. 1485-97. http://doi.org/10.1101/gad.14.12.1485

Jha, P., Ochatt, S.J. and Kumar, V., 2020. *WUSCHEL*: a master regulator in plant growth signaling. *Plant Cell Reports*, 39, pp. 431–444. http://doi.org/10.1007/s00299-020-02511-5.

Karami, O., Aghavaisi, B. and Mahmoudi Pour, A., 2009. Molecular aspects of somatic-to-embryogenic transition in plants. *Journal Chemical Biology*, 2, pp. 177–190. http://doi.org/10.1007/s12154-009-0028-4.

Klaus, F.X., Mayer, H. S., Haecker, A., Lenhard, M., Jorgenes, G. and Laux, T., 1998. Role of *WUSCHEL* in regulation Stem Cell Fate in the *Arabidopsis* Shoot Meristem. *Cell*, 95, pp. 805-815. http://doi.org/10.1016/S0092-8674(00)81703-1

Klimaszewska, K., Pelletier, G., Overton, C., 2010. Hormonally regulated overexpression of Arabidopsis WUS and conifer LEC1 (CHAP3A) in transgenic white spruce: implications for somatic embryo development and somatic seedling growth. *Plant Cell Reports*, 29, pp. 723-734. http://doi.org/10.1007/s00299-010-0859-z

Lara-Lopes, F., Galvan-Ampudia, C. and Benoit L., 2021. WUSCHEL in the shoot apical meristem: old player, new tricks. *Journal of Experimental Botany*, 72(5), pp. 1527–1535, http://doi.org/10.1093/jxb/eraa572.

Laux, T., Klaus, F.X., Mayer, J.B. and Gerd J., 1996. The *WUSCHEL* gene is required for shoot and floral meristem integrity in *Arabidopsis*. *Development*, 122, pp. 87-96. http://doi.org/10.1242/dev.122.1.87

Mayer, K.F., Schoofs, H., Haecker, A., Lenhard, M., Jurgens, G. and Laux, T., 1998. Role of *WUSCHEL* in regulating stem cell fate in the *Arabidopsis* shoot meristem. *Cell*, 95, pp. 805-815. http://doi.org/10.1016/S0092-8674(00)81703-1

Miyazawa, Y., Nakajima, N., Abe, T., Sakai, A., Fujioka, S., Kawano, S., Kuroiwa, T. and Yoshida S., 2003. Activation of cell proliferation by brassinolide application in tobacco BY-2 cells: effects of brassinolide on cell multiplication, cell-cycle-related gene expression, and organellar DNA contents. *Journal Experimental of Botany*, 54(393), pp. 2669-78. http://doi.org/10.1093/jxb/erg312

Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bioassays

with tobacco tissue cultures. *Physiologia Plantarum*, 15, pp. 473-497. http://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nandhakumar, N., Kumar, K., Sudhakar, D. and Soorianathasundram, K., 2018. Plant regeneration, developmental pattern and genetic fidelity of somatic embryogenesis derived *Musa* spp. *Journal of Genetic Engineering and Biotechnology*, 16(2), pp. 587-598. https://doi.org/10.1016/j.jgeb.2018.10.001.

Neelakandan, A.K. and Wang, K., 2012. Recent progress in the understanding of tissue culture induced genome level changes in plants and potential applications. *Plant Cell Reports*, 31, pp. 597–620. http://doi.org/10.1007/s00299-011-1202-z

Nolan T. M., Vukasinovic, N., Liu, D., Russinova, E. and Yin, Y., 2020. Brassinosteoids: multidimensional regulators of plant growth, development and stress responses. *The Plant Cell*, 32(2), pp. 295-318. http://doi.org/10.1105/tpc.19.00335

Noriko, K., Hiroshi, N., Atsushi, M., Yutaka, S. and Makoto, M., 2003. Isolation and characterization of a rice WUSCHEL-type homeobox gene that is specifically expressed in the central cells of a quiescent center in the root apical meristem. *Plant Journal*, 35, pp. 429–441. http://doi.org/10.1046/j.1365-313X.2003.01816.x

Novak, F.J., Arza, R., VanDuren, M., PereaDallos, M., Conger, B.V. and Tang, X., 1989. Somatic embryogenesis and plant regeneration in suspension culture of dessert (AA and AAA) and cooking (ABB) bananas (*Musa* spp). *Nature Biotechnology*, 7, pp. 154–159. http://doi.org/10.1038/nbt0289-154

Okuzaki, A., Konagaya, Ki., Nanasato, Y., Tsuda, M. and Tabei Y., 2011. Estrogen-inducible GFP expression patterns in rice (*Oryza sativa* L.). *Plant Cell Reports*, 30, pp. 529–538. http://doi.org/10.1007/s00299-010-0963-0

Pérez-Hernández, J.B., and Rosell-García, P., 2008. Inflorescence proliferation for somatic embryogenesis induction and suspension-derived plant regeneration from banana (*Musa* AAA, cv. 'Dwarf Cavendish') male flowers. *Plant Cell Reports*, 27, pp. 965–971. http://doi.org/10.1007/s00299-008-0509-x

Ponsamuel, J., Samson, N.P., Ganeshan, P.S., Sathyaprakash, V. and Abraham G.C., 1996. Somatic embryogenesis and plant regeneration from the immature cotyledonary tissues of cultivated tea (*Camellia sinensis* (L). O. Kuntze). *Plant Cell Reports*, 16, pp. 210–214. http://doi.org/10.1007/BF01890869

Schoofs, H., Panis, B., Strosse, H., Mayo-Mosqueda, A., López Torres, J., Roux, N., Dolezel J. y Swennen, R. 1999. Cuellos de botella en la regeneración y mantenimiento de las suspensiones celulares morfogénicas de banano y la regeneración de las plantas vía embriogénesis somática a partir de ellas. *INFOMUSA*, 8(2), pp. 3-6. www.musalit.org/seeMore.php?id=13989

Shoofs, Hilde., 1997. The Origin of Embryogenic cells in *Musa*. *Dissertations of Agriculture*, Ktholieke Universiteit Leuven. Pp. 25-31. http://lib.ugent.be/catalog/rug01:000402461

Shivani, A., Sharma, P., Kaur, V., Kaur, N., Pandey, N., and Tiwari, S., 2017. Genome-wide analysis of transcription factors during somatic embryogenesis in banana (*Musa* spp.) cv. Grand Naine. *PLOS ONE*, 12(8), p. e0182242. http://doi.org/10.1371/journal.pone.0182242.

Sipen, P., and Davey, M. R., (2012). Effects of N(6)-benzylaminopurine and Indole Acetic Acid on In Vitro Shoot Multiplication, Nodule-like Meristem Proliferation and Plant Regeneration of Malaysian Bananas (Musa spp.). *Tropical Life Sciences Research*, 23(2), pp. 67–80. http://www.tlsr.usm.my/tlsr23022012/23022012_07.pdf

Solís-Ramos, L.Y., González-Estrada, T., Nahuath-Dzib, S., Rodríguez-Zapata L.C. and Castaño, E., 2009. Overexpression of

WUSCHEL in *C. chinense* causes ectopic morphogenesis. *Plant Cell Tissue Organ Culture*, 96, pp. 279–287. http://doi.org/10.1007/s11240-008-9485-7.

Sreekala, C., Wu, L., Gu, K., Wang, D., Tian, D. and Yin, Z., 2005. Excision of a selectable marker in transgenic rice (*Oryza sativa* L.) using a chemically regulated Cre/loxP system. *Plant Cell Reports*, 24, pp. 86–94. http://doi.org/10.1007/s00299-004-0909-5

Strosse, H., Domergue, R., Pains, B., Escalant, J.V. and Côte, F., 2003. Banana and plantain embryogenic cell suspensions. INIBAP Technical Guideline 8. In: Vézina A, Picq C (Eds). International Network for the Improvement of Banana and Plantain, Montpellier. Pp. 1-36.

Strosse, H., Schoofs, H., Panis, B., Andre, E., Reyniers, K. and Swennen, R., 2006. Development of embryogenic cell suspensions from shoot meristematic tissue in bananas and plantains (Musa spp.). *Plant Science*, 170, pp. 104–112. http://doi.org/10.1016/j.plantsci.2005.08.007

Weiste, C., Pedrotti. L., Selvanayagam, J., Muralidhara, P., Fröschel, C., Novák, O., Ljung, K., Johannes, Hanson, J., and Dröge-Lase, W., 2017. The Arabidopsis bZIP11 transcription factor links low-energy signaling to auxin-mediated control of primary root growth. *PLoS Genetics*, 13(2), p. e1006607. http://doi.org/10.1371/journal.pgen.1006607.

Winkelmann, T., 2016. Somatic versus zyotic embryogenes: learning from seed. In: Germana, M., Lambardi, M., eds. *In vitro embryogenesis in higher plants*. Methods in Molecular Biology, vol 1359. Human Press, New York, NY. http://doi.org/10.1007/978-1-4939-3061-6_2.

Xu, C.X., Panis, B., Strosse, H., Li, H.P., Xiao, H.G., Fan, H.Z. and Swennen, R., 2005. Establishment of embryogenic cell suspensions and plant regeneration of the dessert banana 'Williams' (*Musa* AAA group). *Journal Horticultural Science Biotechnology*, 80, pp. 523–528. http://doi.org/10.1080/14620316.2005.11511972

Yang, X, and Zhang, X., 2010. Regulation of somatic embryogenesis in higher plants. *Critical Review Plant Sciences*, 29, pp. 36–57. http://doi.org/10.1080/07352680903436291

Youssef, M., James, A., Mayo-Mosqueda, A., Ku-Cauich, J.R., Grijalva-Arango, R. and Escobedo-GM, R.M., 2010. Influence of genotype and age of explant source on the capacity for somatic embryogenesis of two Cavendish banana cultivars (*Musa acuminata* Colla, AAA). *African Journal of Biotechnology*, 9(15), pp. 2216-2223. http://doi.org/10.5897/AJB10.1807

Zhang, Y., Li, H., Ouyang, B., Lu, Y. and Ye, Z., 2006. Chemical-induced auto expression of selectable markers in elite tomato plants transformed with a gene conferring resistance to lepidopteran insects. *Biotechnology Letters*, 28, pp. 1247–1253. http://doi.org/10.1007/s10529-006-9081-z

Zuo, J., Niu, Q.W. and Chua, N.H., 2000. An estrogen receptor-based trans activator XVE mediates highly inducible gene expression in transgenic plants. Plant Journal, 24(2):265–273. http://doi.org/10.1046/j.1365-313x.2000.00868.x

Zuo, J., Niu, Q.W., Frugis, G., Chua, N.H., 2002. The WUSCHEL gene promotes vegetative-to-embryonic transition in *Arabidopsis*. *Plant Journal*, 30(3), pp. 349–359. http://doi.org/10.1046/j.1365-313X.2002.01289.x

Zuo, J., Niu, Q.W., Moller, S.G., Chua, N.H., 2001. Chemical-regulated, site-specific DNA excision in transgenic plants. *Nature Biotechnology*, 19, pp. 157–161. http://doi.org/10.1038/84428