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BASIC AREAS
Article

HD2 overexpression induces dwarfism and elevated histone deacetylase activities in transgenic *Ananas comosus* variety MD2

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ABSTRACT: Plant tissue culture has served as one of the most efficient biotechnological tools to mass produce high quality major crops. Nevertheless, the use of this technique can result in the occurrence of somaclonal variation, which may be brought upon by genetic and/or epigenetic modulations. The epigenetic modulation of plant growth, particularly on the importance of histone deacetylation/acetylation on genome stability, transcriptional regulation, as well as plant development, has been reported in several species such as *Arabidopsis*, rice, and maize. However, the information on its role in regulating the growth of major crops such as pineapple is still lacking. Previously, we had reported that supplementation of high cytokinin in the tissue culture media and exposure to salinity and abscisic acid (ABA) resulted in the production of somaclonal variants that exhibited dwarf phenotypes, and that these occurrences were associated with epigenetic modulation via the involvement of histone deacetylases (HDACs). Thus, in the present study, we aimed to further elucidate and verify the involvement of histone deacetylation in the occurrence of dwarfism in tissue culture-derived pineapple plants, through *HD2* gene overexpression. *AtHD2* gene from *Arabidopsis thaliana* was synthesized and expressed in phenotypically normal *Ananas comosus*. Transformation efficiency was also studied, and application of 200-µM acetosyringone was observed to be more efficient than 500 µM vanillin by 10%. The transgenic plants were observed to exhibit dwarfed phenotypes and showed significantly high HDAC enzyme activities. Ploidy evaluation of the transgenic plants showed no ploidy change had occurred in the samples and remained as 2n. The relative expression level of *AtHD2* was also higher (3.2-fold up-regulation) in the transgenic plants compared to the wild type plants. Collectively, these results indicate that HDACs indeed played an essential role in the response of *A. comosus* towards abiotic stress, and that this phenomenon is epigenetically regulated.

Key words: pineapple, histone deacetylation, transgenic, transformation efficiency, dwarfism, abiotic stress.

INTRODUCTION

Pineapple [*Ananas comosus* (L). Merr.] is one of the most valuable fruits that belong to the family of Bromeliaceae. It has been widely planted in major tropical and sub-tropical regions of the world, mainly for its fruit (Iwuchukwu et al. 2017). Pineapple ranked third for global production after banana and citrus (Paull and Duarte 2011, Joy and Anjana 2016). MD2 variety has dominated most pineapple production and fresh markets in many countries over the Smooth Cayenne, which was a popular variety before MD2 (Reid and Jiang 2011). It has lustrous, green golden shell color, and a regular and appealing cylindrical shape which made it to be known as "golden pineapple" (Janick 2003). In Malaysia, the MD2 cultivar is the leading pineapple variety planted for commercial production, due to its attributes (aroma, sweetness, and color) that attracted the demand by local consumers and international markets (Ankrah and Dorward 2015, Halim et al. 2018). To meet the high demand of MD2 fruits in local and foreign markets, for instance China, Korea, Singapore, and the Middle East, MD2 pineapple has been included in Malaysia's National Agrofood Policy 2.0.

Biotechnological tools offer immense opportunities for improving major crops. For example, in pineapple, genetic transformation via direct gene transfer and *Agrobacterium*-mediated transformation has allowed for small targeted changes to be made in the recipient's genome (Kole and Hall 2008). In addition, pineapple could be improved through somaclonal variation (SV). This phenomenon occurred as a result of tissue culture techniques which yielded in genetic variability that may be useful and valuable for crop improvement (Ebrahimi et al. 2018). In recent years, few studies have detected SV using molecular markers (Aversano et al. 2009, Bairu et al. 2011). SV is sometimes considered as an undesirable by-product due to the stress caused by tissue culture procedures (Mgbeoji and Benda 2016). However, somaclonal manipulations in cultured plant cells also can generate variants that are useful for breeders (Vasil 1988).

Somaclonal variability often emerged in tissue culture due to mechanisms of epigenetic control or changes in the genome of differentiating vegetative cells (Soniya et al. 2001). During de-differentiation and re-differentiation of cells, both qualitative and quantitative changes may occur in the genome, which then resulted in different DNA sequences to be deleted or amplified during cell reprogramming. Somaclonal variation has also been reported to be closely associated with factors such as the source of tissue (explant) and the regeneration system. The changes in chromosome number and structure that occurred during early *in-vitro* induction could be gross or obscure (Mujib et al. 2007). These changes may range from variations in chromosome numbers and structures, to occurrence of chromosomal irregularities including chromosome breaks, fragmentation, deletions, inversions and generation of ring chromosomes (Nwauzoma and Jaja 2013). Such chromosomal irregularities may result in the loss of genes or gene function, as well as the activation or expression of previously silent or recessive genes, when they become haploid (Chen 2007). Chromosome breakage and rearrangements usually occur during *in-vitro* culture techniques (Neelakandan and Wang 2012), whereas chromosome breakpoints usually happened between inter heterochromatic knobs and the centromere (Lee and Phillips 1987) or within centromeric heterochromatin (Johnson et al. 1987). Chromatin modification is controlled precisely by a range of transcriptional regulators that responds to both the cellular and environmental stimuli, hence reducing the proper temporal and spatial development in eukaryotic organisms (Orphanides and Reinberg 2002, Barrera and Ren 2006, Li et al. 2007). These chromatin structures in turn are regulated by post-translational modifications of the histone proteins, which involve various enzymes to modify the histones and chromatin remodeling machines (Probst et al. 2004, Li et al. 2007, Habu 2017).

Plants have unique histone deacetylase (HDAC) enzymes called the HD2-type deacetylases. HDACs often work together with histone methyltransferases (HMTs) and DNA methyltransferases in its action (Bannister and Kouzarides 2011, Vriet et al. 2015). For example, overexpression of *HDAC1* in rice was shown to result in a striking phenotypic change and boost the growth rate of the plant (Yaacob et al. 2013). In *Arabidopsis*, mutations of *HDA6* genes that encode for Rpd3-type HDAC has shown that these genes were involved in gene silencing, while antisense inhibition of HD2-type HDAC resulted in seed abortion (Tanaka et al. 2008). Other examples on HDACs activity have also been observed in other plants, and they imply that HDACs repress gene transcription and hence repress gene expression as well (Pazin and Kadonaga 1997, Ikeuchi et al. 2015, Tang et al. 2017). Perrella et al. (2013) reported that overexpression of HDACs would often produce pleiotropic morphological abnormalities.

Previously, we have reported that the supplementation of high cytokinin concentration (4 $mg{\cdot}L^{-1}$ BAP plus 2 $mg{\cdot}L^{-1}$ IBA), as well as the exposure to salinity and abscisic acid (ABA), resulted in the production of dwarf pineapple variants (Halim et al. 2018). The generated dwarf plantlets exhibited higher HDAC enzyme activity compared to non-dwarf plantlets, and their capability to revert to non-dwarf phenotype suggested that this somaclonal variation occurrence might be due to epigenetic modulation.

Thus, in this study, we aimed to elucidate and verify the involvement of HDAC in the occurrence of dwarfism in tissue culture-derived pineapple plants through *HD2* gene overexpression.

MATERIALS AND METHODS

Plant materials

The leaf base (about 1 cm in length) of *A. comosus* var. MD2 plantlets was excised and used as explants to induce shoot proliferation. The explants were cultured on Murashige and Skoog (1962) media supplemented with 1 $mg{\cdot}L^{-1}$ IBA and 2 $mg{\cdot}L^{-1}$ BAP, 3% sucrose and 2 $g{\cdot}L^{-1}$ Gelrite for two months. For transformation experiments, the leaf base of the regenerated

in-vitro plantlets (approximately 1 cm in length) was longitudinally dissected to produce two explants upon transformation. All cultures were maintained in the incubation room at 25 ± 1°C under a photoperiod of 16/8 h light/dark conditions with light illumination of 1,000 lux.

Synthesis of *HD2* gene and plasmid construct

The *HD2* gene from *Arabidopsis thaliana*, *AtHD2* (based on The Arabidopsis Information Resource, accession no. 4010741337; locus tag: AT5G22650) was synthesized and inserted into pCambia 1304 plasmid by Next Gene Scientific Sdn. Bhd. (Kuala Lumpur, Malaysia) before the plasmid construct (Fig. 1) was transformed into *Agrobacterium tumefaciens* cells.

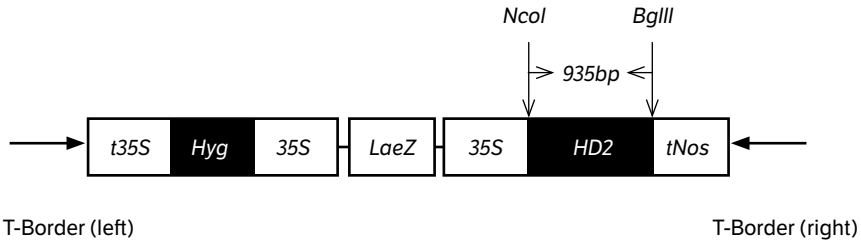


Figure 1. Plasmid construct of pCambia 1304 harboring the *HD2* gene.

Preparation of *Agrobacterium tumefaciens* for *Ananas comosus* transformation

Agrobacterium cells containing the gene cassette were revived from a glycerol stock by growing on yeast extract beef (YEB) medium (Oxoid, England) supplemented with 50 mg·L^{-1} rifampicin (Duchefa Biochemie, The Netherlands) and incubated at 28°C for two or three days. A single colony was picked and allowed to grow in a 5 mL YEB medium containing 50 mg·L^{-1} rifampicin, on an incubator shaker (120 rpm) at 28°C overnight. Ten mL of YEB media was added to 4 mL overnight culture and further incubated at 28°C for 4 hours (120 rpm). The culture was then centrifuged at 10,000 rpm for 5 min to harvest the cell pellet, and its supernatant was discarded. The pellets were re-suspended in 50 µL Luria-Bertani (LB) media before plating on LB media supplemented with 50 mg·L^{-1} streptomycin (Sigma, United States of America) and 50 mg·L^{-1} kanamycin (Sigma, United States of America). The cultures were incubated at 28ºC.

Verification of the presence of *AtHD2* in *Agrobacterium tumefaciens* by colony PCR

Successful integration of *AtHD2* gene into the pCambia 1304 plasmid was verified by colony polymerase chain reaction (PCR) before *A. comosus* transformation was carried out. All single colonies grown on the selection plate were subjected to colony PCR following standard method, as shown in Table 1. The PCR reagents used were listed in Table 2, while *gfp* primers (Table 3) were used to verify the successful integration of the gene and its presence in the *Agrobacterium* cells.

Table 1. Polymerase chain reaction thermal cycling protocol to amplify *gfp* and *HD2* genes.

Steps	Temperature (˚C)	Time	Cycle
Initial denaturation	94	2 min	1
Denaturation	95	30 s	
Annealing	47-57	60 s	30
Extension	68	1 min	
Final extension	72	7 min	1
Cooling	10	Indefinite	1

Table 2. Polymerase chain reaction (PCR) reagents for *gfp* and *HD2* gene amplification.

PCR reagents	Volume (µL)	Final concentration
10 X buffer	2.5	1x
25 mM $MgCl_2$	3.5	2.5 mM
dNTP	1	0.2 mM
Upstream primer	1	0.5 mM
Downstream primer	1	0.5 mM
Taq DNA polymerase 5 U/µL	0.1	1.25 U
Template DNA	1	0.5 µg/µL
Sterile double distilled water	14.9	-
Total volume	25	-

Table 3. *gfp* and *HD2* gene specific primers used for sequence amplification.

Type of primers	Forward sequence (5'- 3')	Reverse sequence (5'-3')	Product size
gfp	CTGGTCGAGCTGGACGGCGACG	CATGGTCCTGCTGGAGTTCGTG	716 bp
HD2	TTACCATGGAATGGAGTTCTGG	GAAGATCTCTTAAGCTCTACCCTT	940 bp

Verification of the sequence orientation by restriction enzyme digestion

Two types of restriction enzyme (RE), *NcoI* and *BglII*, were used to verify the orientation of the sequence after gene insertion. The component for RE digestion is shown in Table 4. The first digestion was carried out using RE with the lowest salt concentration, NcoI-HF. The reaction was incubated at 37°C for 5-15 min. Sufficient NaCl (100 mM) was added according to the manufacturer's protocol (NEB, England). About 10 units or 1 µL of *BglII* then were added and incubated at 37°C for 5-15 min.

Table 4. The components and reactions protocol used for restriction enzyme digestion.

Components	50 µL reaction
DNA	up to 1 µg
10X CutSmart buffer	5 µL (1X)
NcoI-HF	1 µL (or 10 units)
Nuclease-free water	to 50 µL

Ananas comosus transformation

Agrobacterium cultures (5 mL) containing the pCambia 1304-*HD2* were grown in LB broth overnight before mixing with fresh 45 mL LB broth containing 100 µg·mL^{-1} streptomycin and 50 µg·mL^{-1} kanamycin. The cultures were placed on an incubator shaker at 28°C, 120 rpm, until the OD600 reached 0.8-1. The cells were harvested by centrifugation at 5,000 rpm for 10 min. The pellet was re-suspended in MS medium and re-shaken in an incubator until the OD600 reached 0.8-1. The leaf base explants were immersed in 50 mL MS medium containing the *Agrobacterium* for 10 min. After infection, the explants were blot dried on a sterilized tissue paper. The explants were then transferred onto solid co-cultivation medium containing 1 mg·mL^{-1} IBA, 2 mg·mL^{-1} BAP, and 200 µM acetosyringone or 500 µM vanillin (in separate experiment) for three days under dark condition. All 100 infected explants were incubated under light until new shoot tips emerged or regenerated. The shoot tips were transferred to selection medium supplemented with 20 mg·mL^{-1} hygromycin (Sigma, United States of America), 250 mg·mL^{-1} cefotaxime (Sangon Biotech, China), and 100 mg·mL^{-1} timentin (Sangon Biotech, China) for shoot regeneration.

Verification of successful transformants containing *HD2* gene

After transformation was conducted, the presence of the gene in the putative transformants was verified by PCR amplification of *HD2* gene. The primer's sequences used are as shown in Table 3. PCR components and reaction protocol were shown in Table 1 and Table 2, respectively.

Quantitative RT-PCR analysis

Extraction of RNA, cDNA synthesis and RT-PCR analysis were conducted based on previously described protocols (Halim et al. 2018). To summarize, the total RNA was extracted from approximately 100 mg of leaf samples by using RNeasy Plant Mini kit (Qiagen, Germany), following the manufacturer's protocol. Then, the elution of RNA was done in 50 µL RNase-free water, and any remaining genomic DNA was removed using RQ1 RNase-free DNase (Promega, United States of America), in which 20 µL of the RNA sample was subjected to DNase digestion in 30 µL reactions. Then, cDNA synthesis was conducted for all DNase-digested RNA samples using RevertAid Reverse Transcriptase (Thermo Scientific, United States of America), according to the manufacturer's protocol for First Strand Synthesis. Following that, real-time PCR (qPCR) was conducted using SensiFAST™ SYBR® Hi-ROX Kit (Bioline, United Kingdom) according to the manufacturer's protocol in an Applied Biosystems StepOne Real-Time PCR System (Thermo Fisher Scientific, United States of America), and the results were analyzed using StepOne Software v2.3 (Thermo Fisher Scientific, United States of America). Optimization of the quantitative RT-PCR experiments was done as previously reported (Halim et al. 2018). The data obtained were normalized to the geometric means of two reference genes (Table 5) and analyzed based on $2^{-\Delta\Delta CT}$ method (Vandesompele et al. 2002). The reference genes used in this study were 18sRNA and β-actin, with NCBI Accession Number: JN129389 and HQ148720, respectively. The sequence of the primer for the HDAC gene was designed based on *A. comosus* HDAC 2 gene (NCBI Accession Number: OAY83945.1). The primers were designed using Primer Premier 6 and were manufactured by IDT Technologies (IDT, United States of America). Table 5 lists the primers used in this analysis (Halim et al. 2018).

Table 5. Primer sequences used in the gene expression analysis.

Gene	Primer sequences
HD2-F	GAGAGACCGTCAAGTGCGAC
HD2-R	AGCCAAGGAAGTAGACGCTG
18srRNA-F	ATGGTGGTGACGGGTGAC
18srRNA-R	CAGACACTAAAGCGCCCGGTA
β-actin-F	CTGGCCTACGTGGCACTTGACTT
β-actin-R	CACTTCTGGGCAGCGGAACCTTT

Flow cytometry analysis

Nuclear isolation and flow cytometry analyses were carried out based on the method described by Doležel et al. (2007). In brief, 1 mL of ice-cold nuclei isolation buffer (LB01) was added to approximately 20 mg of leaf tissues in a petri dish. Then, the leaf tissues were immediately chopped using a sterile razor blade attached to a sterile scalpel. After that, the resulting homogenate was gently mixed several times via gentle pipetting, followed by filtration using a 4 µm nylon mesh, before being added with DNA fluorochromes (50 $\mu g \cdot mL^{-1}$ propidium iodide; Sigma-Aldrich, United States of America) and 50 $\mu g \cdot mL^{-1}$ RNase (Sigma-Aldrich, United States of America). The samples were gently mixed and incubated for 10 min on ice. The mixture was then injected into a FACS Calibur Flow Cytometer (Becton Dickinson, United States of America) for ploidy analysis and nuclear DNA estimation (Doležel et al. 2007). The reference standard used in this study was soybean (*Glycine max* cv Polanka) leaves with a known ploidy number (2n) and genome size (2.50 picogram) (Doležel et al. 2007).

Nuclear protein extraction and Bradford assay

The transformed and non-transformed plantlets were analyzed for their HDAC activities using phenotypically normal plantlets as the control. Nuclear protein was extracted from all samples using Plant Nuclei Isolation/Extraction kit (Sigma-Aldrich, United States of America). The bovine serum albumin (BSA, Bio-Rad, United States of America) standard curve was prepared by using Quick Start™ Bradford Protein Assay (Bio-Rad, United States of America). The concentration of the nuclear proteins were then quantified using Bradford's reagent based on a 96-well plate assay protocol (Halim et al. 2018). The absorbance values were read at 594 nm using Sunrise™ microplate reader (Tecan, Austria). The concentrations of the protein in the samples were then determined based on the standard curve.

Histone deacetylase enzyme activity analysis

The HDAC enzyme activity of the samples was measured using EpiQuik™ HDAC Activity or Inhibition Assay Kit (colorimetric), following the manufacturer's protocol (EpiGentek, United States of America). Acetylated histone substrate was coated on the strip wells of a 96-well microplate. Then, 10 µg of nuclear protein extracts and assay buffer (49 µL) were added to the acetylated substrate and incubated at 37°C for 90 min. Incubation would allow for the active HDACs present in the nuclear protein extracts to bind and effectively remove the acetyl groups from the substrate. Then, 150 µL wash buffer was used to wash the wells before 50 µL capture antibody was added, and the wells were washed again and added with 50 µL of detection antibody solution. Subsequently, the developing solution was added for color development, and the amounts of deacetylated products were colorimetrically quantified by measuring the absorbance at 450 nm, using Sunrise™ microplate reader (Tecan, Austria). Following that, a deacetylated histone standard curve was prepared and used to calculate the amount of deacetylated product (ng) and HDAC enzyme activity of the samples. The Eqs. 1 and 2 were used for the calculation:

$$\text{Deacetylaed product (ng)} = \frac{\text{(Sample OD-Blank OD)}}{\text{Slope}} \quad (1)$$

$$\textit{HDAC}\text{ activity ((ng/min)/mg)} = \frac{\text{Deacetylated product (ng)}}{\text{protein amount (µg) x incubation time (min)}} \text{ x } 1{,}000 \quad (2)$$

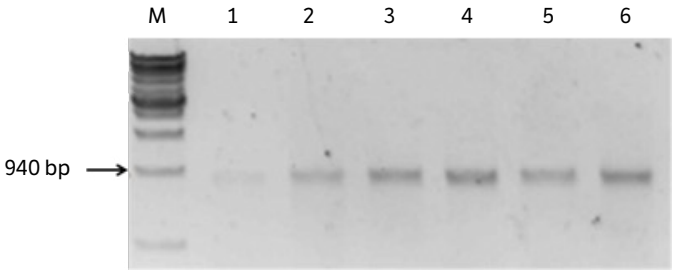
Statistical analysis

All experiments were conducted following a randomized complete block design (RCBD) in triplicates. Statistical analysis was done by either t-test or one-way analysis of variance (ANOVA), and the mean values were separated using Duncan's multiple range test (DMRT) at 5% significance level using Statistical Package for the Social Sciences (SPSS) version 22.

RESULTS AND DISCUSSION

Verification of *AtHD2* gene in the plasmid of *Agrobacterium tumefaciens*

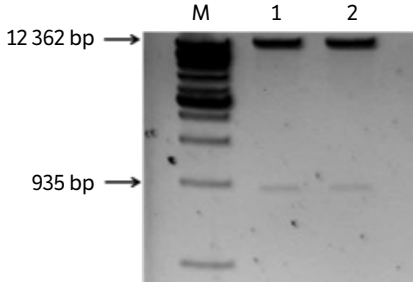
In this study, *AtHD2* gene cassette was designed and transformed into *A. tumefaciens*. The *A. tumefaciens* cultures were maintained and grown on a selection media to produce single colonies. Five successfully formed colonies were screened by colony PCR to confirm the plasmid vector harboring the *AtHD2* gene. The orientation of the plasmid construct was then determined using RE digestion followed by gel electrophoresis. This was carried out prior to *AtHD2* gene transformation into *A. comosus*. The optimum annealing temperature (AT) was identified at 50.8°C. As shown in Fig. 2, the *AtHD2* gene primer pair used in this study has amplified the target gene with an approximate size of 940 bp, indicating successful transformation of the gene in the plasmid construct of *A. tumefaciens*.



*Lane M: 1 kb DNA markers (Vivantis Technologies Sdn Bhd, Malaysia); lane 1: amplified DNA with AT of 49°C; lane 2: amplified DNA with AT of 49.3°C; lane 3: amplified DNA with AT of 49.9°C; lane 4: amplified DNA with AT of 50.8°C; lane 5: amplified DNA with AT of 52.1 °C; lane 6: amplified DNA with AT of 53.4°C.
Figure 2. Gel image showing DNA bands obtained after gradient polymerase chain reaction amplified with *HD2* gene primers. The most intense band on lane 4 was obtained with annealing temperature (AT) of 50.8°C.

Verification of the orientation of the plasmid construct

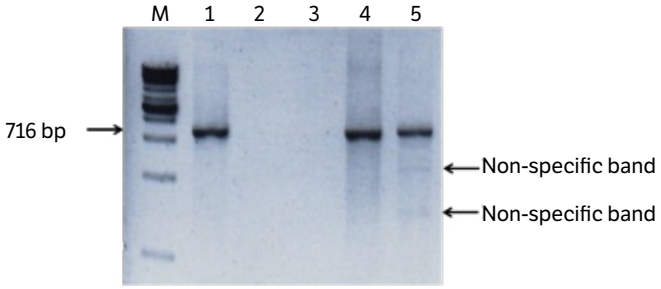
RE (*NcoI* and *BglII*) digestions were carried out to ensure the correct plasmid construct orientation and no formation of sequence mutation in the plasmid vector. Figure 3 shows two fragments from the RE digestion, in which the DNA bands observed indicated the correct sizes of both plasmid backbone (~12 362 bp) and DNA insert (~935 bp).



*Lane M: DNA markers 1 kb (Vivantis Technologies Sdn Bhd, Malaysia); lane 1: DNA bands cut by *NcoI* and *BglII* for plasmid extracted from colony; lane 2: DNA bands cut by *NcoI* and *BglII* for plasmid extracted from colony 4.
Figure 3. Gel showing DNA bands obtained after restriction enzymes digestion.

Confirmation of *gfp* gene in the plasmid construct

From the five colonies formed, only three showed positive bands using *gfp* primers, as shown in Fig. 4. The primer used has amplified the green fluorescence (*gfp*) gene resulting in a PCR product size of 716 bp. However, lane 5 showed the presence of additional non-specific bands. For subsequent transformation experiments, only colonies with specific bands (lanes 1 and 4) were used.

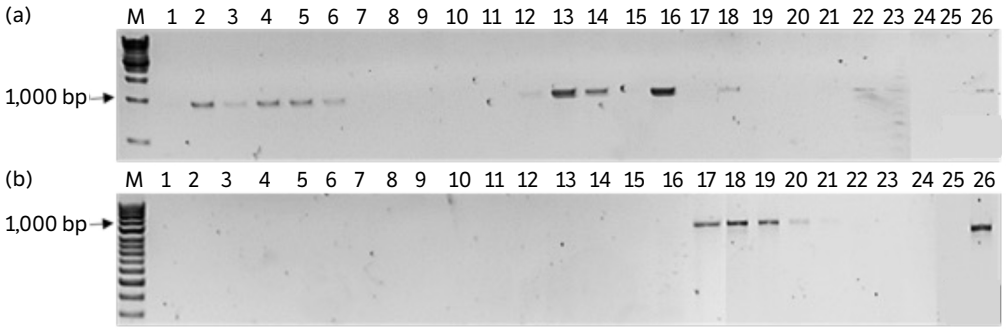


*Lane M: 100 bp DNA markers (Vivantis Technologies Sdn Bhd, Malaysia); lane 1: amplified DNA band showing positive/successful transformation from *Agrobacterium* colony 1; lane 2: no DNA band showing negative/unsuccessful transformation from an *Agrobacterium* colony 2; lane 3: no DNA band showing negative/unsuccessful transformation from an *Agrobacterium* colony 3; lane 4: amplified DNA band showing positive/successful transformation from an *Agrobacterium* colony 4; lane 5: amplified DNA band showing positive/successful transformation from an *Agrobacterium* colony 5 with unspecific bands.
Figure 4. Gel showing amplified DNA fragments from each colony by polymerase chain reaction.

Expression of *AtHD2* in transgenic *Ananas comosus* and transformation efficiency using acetosyringone and vanillin

Three cycles of genetic transformation were carried out to determine the role of *HD2* gene in the generation of somaclonal variants. Thus, to test the function of the gene, the synthesized *AtHD2* gene was first inserted into pCambia 1304 plasmid vector and transformed into the *A. comosus* plant system using *A. tumefaciens*. Transformation by *A. tumefaciens* is more efficient compared to other techniques, as it resulted in the transgenes to be in a more stable manner in the plant system. However, the lack of signaling molecules such as phenolic compounds in monocot plants including pineapple (*A. comosus*) hampers the success rate to transfer foreign genes (Yadav and Singh 2013). Thus, the application of widely used phenolic compounds such as acetosyringone, cinnamic acid, coumaric acid, and vanillin are crucial for transformation of monocot plants such as rice, wheat, pineapple, and others (Yadav and Singh 2013). To date, most *A. tumefaciens*-mediated transformation protocols of dicot and monocot plants rely on exogenous addition of phenolic compounds to enhance the transformation events, since these compounds are not naturally produced (Yadav and Singh 2013).

Acetosyringone is well known for its function in promoting high efficiency transformation in various plants as it functions to induce the *Agrobacterium*-vir genes, thus enabling the transfer of T-DNA into the host plants (Subramoni et al. 2014). On the other hand, 500 µM of vanillin has been reported to improve the transformation efficiency of *A. comosus* variety N36 (Aziz et al. 2012)[3]. Thus, the efficiency of acetosyringone and vanillin in aiding transformation of *HD2* gene in *A. comosus* variety MD2 was compared in this study. As observed in Fig. 5, transformation using acetosyringone as the signaling molecule was more efficient and resulted in generation of more transgenic lines than vanillin.



*Lanes 25 and 26 are negative and positive control, respectively; M in (A) consists of 1 kb DNA marker (Vivantis, Malaysia), while M in (B) consists of 100 bp DNA marker (Genesta™).

Figure 5. Gel electrophoresis images showing putative transgenic lines (amplified using *HD2* primer) producing 940 bp polymerase chain reaction product size. The plants were transformed using (a) acetosyringone, and (b) vanillin.

Moreover, data analysis also revealed that addition of acetosyringone (200 µM) improved the transformation efficiency by 10%, compared to vanillin (500 µM) (Fig. 6). Similar observations were obtained in previous studies, such as in almond transformation, in which the presence of 150 µM of acetosyringone has resulted in 12.3% transformation efficiency (Costa et al. 2006). In a study conducted by Wu et al. (2003), 200 µM of acetosyringone applied during wheat transformation has increased the efficiency by 3.3%. Acetosyringone has also been used in transformation of pineapple. For example, use of 100 µM of acetosyringone was reported to result in 20.6% transformation frequency in pineapple (Gangopadhyay and Mukherjee 2015), however this value is much lower than that obtained in this study. On the other hand, it has been reported that the addition of 500 µM vanillin yielded 55.5% of transformation efficiency in transgenic N36 pineapple (Aziz et al. 2012)[3], considerably higher than that obtained in this study (33.3%).

Vanillin has also improved the *Agrobacterium*-mediated transformation for microalgae (Pratheesh et al. 2014). It has been reported that the success and efficiency of plant transformation vary immensely according to the plant species, the genotype, and the plant tissue types that are used.

[3] Aziz, A., Hamzah, M.-I. and Cha, T.-S. (2012). News from Malaysia. Newsletter of the Pineapple Working Group, International Society for Horticultural Science Issue No 19, 43.

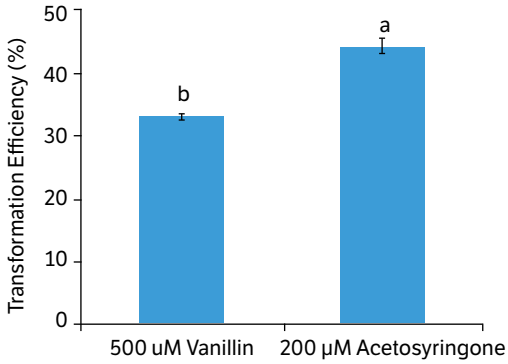
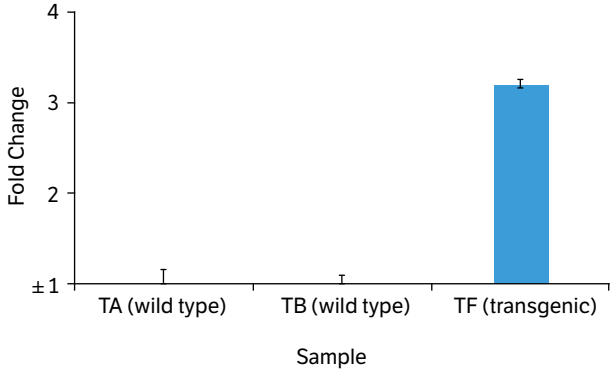


Figure 6. The percentage of transformation efficiency for two types of phenolic compounds: vanillin (500 µM) and acetosyringone (200 µM). The transformation efficiency was calculated from the number of putative transgenic lines (survived plantlets) over the total number of samples inoculated on the selection media.

Gene expression analysis of *AtHD2* and morphology of transgenic *Ananas comosus*

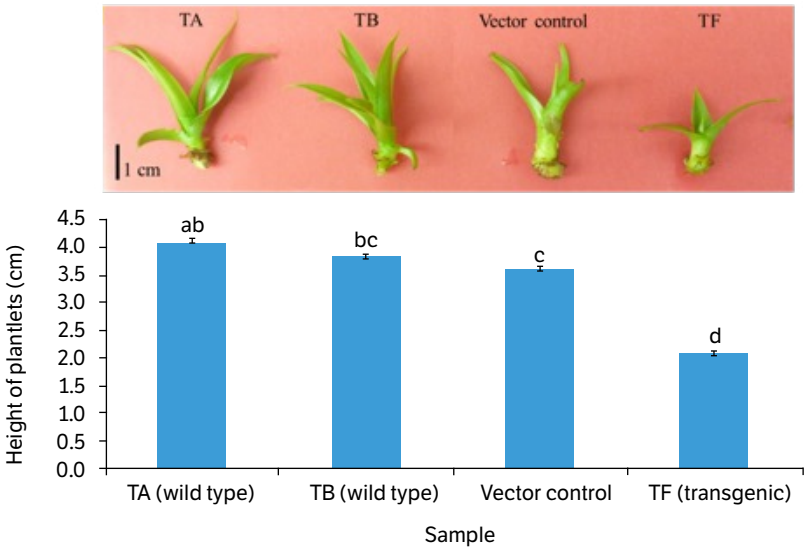
The transformation of *A. thaliana* HDAC gene, *AtHD2* into *A. comosus* plantlets, was studied to determine the relative expression level of the gene and to verify its function in the occurrence of somaclonal variation. Data analysis revealed a higher relative expression of *AtHD2* (up-regulated by 3.2-fold) in the transgenic sample (TF) compared to the control/wild type (TA and TB) (Fig. 7). This reflected a successful transformation of *AtHD2* in *A. comosus*. Moreover, upon transformation of the *AtHD2* gene, the putative transgenic plantlets were allowed to grow on the selection media containing hygromycin for about three months preceded by observation of height morphology. As observed in Fig. 8, the height of the transgenic plantlet (about 2 cm) was significantly shorter than the wild types (about 4 cm).

It has been reported that the *AtHD2A*, *AtHD2B*, and *AtHDC* (homologies of *Arabidopsis* HDAC gene) were associated with diverse developmental aberrations such as early senescence, ectopic expression of silenced gene, apical dominance disturbance, homeotic imbalance and flower defects (Tian and Chen 2001). Furthermore, *AtHD2* expression has been observed to cause development of a short silique, abortion of seeds, created a mutant phenotype with narrowing and curling of leaves and slow down blooms (Lagacé et al. 2003, Han et al. 2016). Briefly, the *AtHD2* expression has been identified to be associated with phenotype instability, thus verified its effects in generating somaclonal variants (dwarf phenotype), as previously shown through supplementation with high cytokinin and exposure to salinity and ABA (Halim et al. 2018).



TA: Plantlets grown on MS basal media; TB: plantlets grown on optimum regeneration media.

Figure 7. Gene expression of *Arabidopsis thaliana* histone deacetylase 2 (*AtHD2*) in the transgenic samples (TF). The fold change was represented by $2^{\wedge -\Delta\Delta CT}$, in which ΔΔCT is the difference between reference ΔCT with target ΔCT.



TA: Plantlets grown on MS basal media; TB: plantlets grown on optimum regeneration media.

Figure 8. Comparison of plantlet height of transgenic plantlets (TF) with wild type plantlets (TA and TB), and vector control (transgenic with an empty vector). The images on the top of the bar chart shows the plantlets from each treatment. Bar = 1 cm.

HDAC enzyme activity of transgenic *Ananas comosus*

Measurement of HDAC enzyme activity of the transgenic plantlets was conducted to determine the activity of HDAC enzyme in the transgenic samples. The HDAC enzyme activity in the transgenic samples was found to be higher compared to the control (Table 6). Histone deacetylation activities were found to be involved in many plant metabolic pathways, including signaling pathways in response to environmental stresses, transcriptional co-activator, protein folding, and various enzymatic reactions (Fisher and Franklin 2011). Based on the results shown in Table 6, the HDAC enzyme activity level of the transgenic plants (TF) was observed to be significantly higher (5,902 ng/min/mg) compared to the wild type TA (666.67 ng/min/mg) and wild type TB (4,370.22 ng/min/mg). These revealed that the transformation of the plantlets with an exogenous *Arabidopsis HD2* gene (*AtHD2*) had resulted in the formation of dwarfed plantlets, with significantly higher HDAC activity levels. These results further confirmed that the occurrence of somaclonal variation (dwarfism) observed in this study was due to HDAC events. It has been reported that the *HD2* gene is involved in regulating the compaction of nucleosomes and therefore gene expression, in which deacetylated histones are associated with subsequent decrease in gene transcription.

Table 6. Histone deacetylase (HDAC) enzyme activity of transgenic and phenotypically normal plantlets (wild types)*.

Sample name	Phenotype	HDAC activity (ng/min/mg)
TA (wild type)	Normal	666.67 ± 22.07^{a}
TB (wild type)	Normal	$4{,}370.22 \pm 46.55^{b}$
TF (transgenic)	Dwarf	$5{,}902 \pm 25.10^{c}$

*Means with different letters in the same column are significantly different at $p < 0.05$ according to analysis of variance, and Duncan's multiple range test; TA: plantlets grown on MS basal media; TB: plantlets grown on optimum regeneration media; TF: transgenic sample.

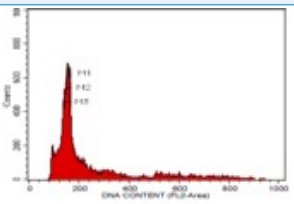
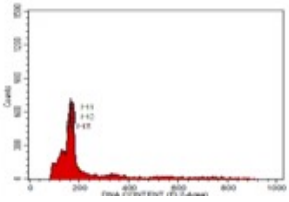
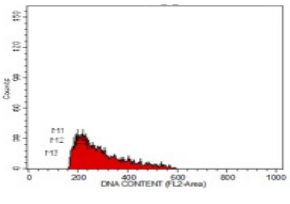
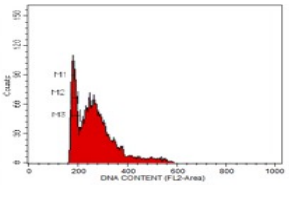
Histone acetyltransferases (HATs) and HDACs have been identified as transcription modulators and have captured the interest of molecular biologists to investigate their roles and involvement in the regulation of gene expression (Lusser et al. 2001). *HDA6* gene from a family of HDAC known as *Reduced Potassium Dependency3* (RPD3) has been reported to mediate histone deacetylation through transcriptional gene silencing (TGS) caused by jasmonic acid and ethylene in *A. thaliana* (Chinnusamy and Zhu 2009). Environmental strains and endogenous signals can repress the expression of target

genes through reduction in histone acetylation levels. For example, the overexpression of *HDA19* gene from the same *RPD3* family in transgenic *A. thaliana* has been observed to decrease histone acetylation levels and increase deacetylation (Zhou et al. 2005). In rice, the expression of *OsNAC6* (a NAC-type transcription factor gene) has been reported to be induced by both biotic and abiotic stresses, in which the overexpression of this gene would result in growth stunting (Nakashima et al. 2007). Interestingly, the expression of this gene was reported to be epigenetically regulated by HDAC *OsHDAC1* (Chung et al. 2009). These observations are in line with our findings, which showed that the response to both biotic and abiotic stresses (such as high cytokinin concentration, exposure to salinity and ABA) are epigenetically modulated through interactions of various factors, especially through the involvement of HDACs, whose function is to restrict plant growth (Halim et al. 2018). This is further strengthened by the observations recorded in this study, which showed that over-expression of *HD2* in *A. comosus* resulted in growth stunting (dwarfism).

Ploidy analysis of transgenic *Ananas comosus*

Ploidy level changes in plants have been reported to be associated with chromosomal aberration and gene mutation that affect the growth of plants (Olhoft and Phillips 1999). Thus, a ploidy analysis was also carried out in this study to investigate if the phenotype abnormality of the transgenic sample was also accompanied by a ploidy change. Data analysis revealed that the ploidy level of the transgenic plantlets was observed to be the same with that of the wild type plants (Table 7). This shows that no chromosomal aberrations were caused by expression of *AtHD2* gene in the transgenic plants. Thus, the variation observed in this study was postulated to be epigenetically controlled through HDAC involvement.

Table 7. Determination of ploidy number and 2C DNA content for transgenic and wild type samples.

Sample	Phenotype	Histogram	2C DNA content (pg DNA)	Ploidy level
Wild type	Normal		2.40 (2.347×10^9 bp)	2n
Wild type	Normal		2.59 (2.533×10^9 bp)	2n
Transgenic	Dwarf		2.30 (2.251×10^9 bp)	2n
Reference standard	28.14a		2.50 (2.445×10^9 bp)	2n

CONCLUSION

In recent years, increasing number of reports can be found on the importance of histone deacetylation/acetylation on the growth and development of several model plants such as *Arabidopsis*, rice, and maize. However, much is yet to be discovered on its role in the growth of major crops such as pineapple. In order to bridge this research gap, a genetic transformation of exogenous *AtHD2* gene in phenotypically normal *A. comosus* plants has been carried out in this study.

The *AtHD2* expression in the transformants was found to be 3.2-fold higher than the wild type plants, which was then translated to significantly elevated HDAC enzyme levels in the samples. The growth of the transformants were also observed to be stunted (dwarfed), possibly due to gene repression brought upon by high *HD2* gene expression. These further supported our earlier findings, which showed that the occurrence of stress-induced somaclonal variation (through supplementation with high cytokinin and exposure to salinity and ABA) was epigenetically modulated through the involvement of HDACs. However, more research is needed to further elucidate the intrinsic mechanism of HDAC, its relationship with other epigenetic modulators and how they respond to stress stimuli.

AUTHORS' CONTRIBUTION

Conceptualization: Yaacob, J. S. and Khalid, N. **Formal Analysis:** Halim, N. A. A., Tan, B. C., Khalid and Yaacob, J. S. **Writing – Original Draft:** Halim, N. A. A., Tan, B. C. and Yaacob, J. S.

DATA AVAILABILITY STATEMENT

All data analyzed during this study are included in this published article.

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