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Identification of Differential-Expressed Genes in Banana-Biostimulant Interaction Using Suppression Subtractive Hybridization

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Abstract: The use of environmental-friendly agricultural inputs to increase yield in crops could reduce environmental contamination and alleviate (a)biotic stress. In this study, we applied a foliar liquid biostimulant to banana plants, which was developed from the anaerobic fermentation of microorganisms with organic remains (manure and plant waste) and minerals to use as environmental-friendly product. To determine the effect of this biostimulant in the plants, a Suppression Subtractive Hybridization (SSH) library was developed from banana leaves in greenhouse conditions. Furthermore, salicylic and jasmonic pathways activation was evaluated by gene expression analysis. First, the application of the biostimulant to banana leaves increased the membrane stability index (MSI), which is directly related to a higher CO_2 exchange and assimilation rate. Additionally, the total chlorophyll content increased in plants in comparison with the control plants. In addition, the SSH analysis revealed around 300 expressed sequence tags (ESTs). Several induced genes associated with different molecular pathways, including photosynthesis, anthocyanins production, and plant defense, among others, were encountered after bioinformatic analysis. Upregulated gene expression was demonstrated in different time points, after biostimulant application. Furthermore, induced expression of genes related to the jasmonic acid pathway was observed, which may prove beneficial to the plant under stress conditions.

Keywords: SSH; pathogen-resistance related genes; salicylic acid; jasmonic acid

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1. Introduction

In 2019, Ecuador was one of the highest world exporters of bananas with $3.43b, followed by the Philippines with $1.93b, Costa Rica with $1.18b, then Guatemala with $1.16b, and finally Colombia with $997m. In Ecuador, banana, cacao, rice, maize, and oil palm represent five of the most harvested crops in the country. Banana production totaled 87.14% of the national exports, which represents 36.6% of the banana world export market [1]. Ecuador is the fourth largest producer of bananas worldwide, and Ecuadorian bananas possess the best world acceptance as a dessert fruit, placing Ecuador as one of the most important producers in the world [2]. Furthermore, the internal consumption of bananas and plantains is increasing. As with many other crops, banana production could be affected by (a)biotic stress including salinity, drought, or fungus infection. Several reports indicate that the application of biostimulants in crops could enhance tolerance to abiotic stress, although other authors also indicate that biostimulants could be used for the

control of biotic stress (reviewed by Ganugi [3]). Abiotic stress in plants could be caused by different conditions in the environment that affect the desired growth and yield of the crops [4,5]. The physiological effect of abiotic stress in plants is the increment of reactive oxygen species (ROS) resulting in the leakage of electrons in cellular compartments, leading to oxidative stress (reviewed by Cataldo et al. [5]). The use of biostimulants could activate anti-oxidative enzymes for the inactivation of ROS in plants [6]. Furthermore, different reports have indicated that the application of biostimulants could improve plant characteristics including nutritional quality [7], photosynthesis [8], secondary metabolites [9], leaf pigments [10], and root growth [11]. Additionally, plants applied with biostimulants are more tolerant to abiotic stress, including salt and drought [12]. Furthermore, the growth of other microorganisms, including rhizobacteria, in plants were encountered [13]. Although biostimulants have been linked to tolerance to abiotic stress in plants, several reports have indicated that the application of biostimulants in plants could reduce the infection of pests and diseases including soil-borne diseases, nematodes, fungi, and viral and bacterial pathogens [14,15].

The major problem in banana production is related to pathogen control. In the 1950s, the most dangerous pathogen reported in Ecuador was *Fusarium oxysporum* f. sp. *cubense* race 1, which devastated Ecuador's most important banana cultivar ('Gros Michel'). This cultivar was replaced by banana cultivars from the Cavendish subgroup from the AAA genotypes [16]. On the other hand, black sigatoka disease is still one of the most critical diseases to be controlled. The ascomycete fungus *Pseudocercospora fijiensis*, which is the causal agent of the disease, was reported in Ecuador since the 1980s. The control of this pathogen increases crop production costs by up to 40%, and the consequences of the intense use of chemical products decreases the effectivity against the pathogen. Thus, the number of applications per year must be incremented to have the same control against the pathogen, resulting in increased environmental pollution and chemical molecules residues for a long period on the applied areas [17].

Efforts for the transition to a sustainable and environmentally friendly agriculture have increased recently, demanding organic amendments or biological fertilizers as an alternative for agrochemicals [18]. Conventional agriculture, where chemical fertilizers and pesticides (agrochemicals) are applied to increase crop productivity, could cause imbalance to the ecosystem due to synthetic chemicals which are applied to the plants and soil [18]. Therefore, a sustainable and environmentally friendly agriculture could be obtained by using less agrochemicals by replacing them with organic alternatives including biostimulants [19]. The concept of biostimulants, which could refer to the use of living microorganisms capable to enhance plant health, relies on the use of environmental-friendly products in crop production. A biostimulant could be conceptualized as a substance or microorganism applied to the plant with the capacity of fertilizing it, increasing plant response to biotic or abiotic stress, or even improving field traits [19]. This biostimulant could be categorized based on the composition or the main effect in seven categories as described [19]. One of the categories include "humic and fulvic acids", which are natural components that could be developed from decomposition of plant through the aid of microorganisms. The high concentration of the microbiome content in this kind of biostimulant has been reported to improve plant productivity. Microorganisms present in biofertilizers (which is a subcategory of biostimulants by increasing nutrient use efficiency by opening novel routes of acquisition by plants according to du Jardin [19]) could produce plant hormones including indole acetic acid (IAA), gibberellins (GA), and cytokinins (CK) (reviewed by Bhardwaj et al., [18]). Research on the effect of biostimulants in crops have been reported at the molecular level, revealing that different pathways are triggered at the cellular and gene level (review by Baltazar et al. [20]).

The use of fermentation of biological wastes in agriculture through microorganisms in anaerobic conditions generates a biostimulant, which is produced at a low cost on-farm [21]. Several authors have described the concept for a biostimulant (reviewed by Cataldo et al. [5]). For instance, the use or not of microorganisms (beneficial or pathogenic)

in crops by the application directly to plants or to the soil is aimed to increment nutrient content, tolerance to (a)biotics stress, and/or to increase crop quality (reviewed by Ganugi [3]). According to Rouphael et al. [22] the biostimulant concept under the EU regulation 2019/10009 is indicated as follows: "A plant biostimulant shall be an EU fertilising product, the function of which is to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: (i) nutrient use efficiency, (ii) tolerance to abiotic stress, (iii) quality traits, or (iv) availability of confined nutrients in the soil or rhizosphere".

Differential gene expression was encountered in plants after biostimulant application [7,23] under abiotic [8,24–27] and biotic [28] stress. Furthermore, an induced expression of genes related to plant growth and defense against pathogens was detected [23]. The hypothesis of this study is the detection of differential-expressed genes after biostimulant application, and the identification of genes related to plant growth and response to (a)biotic stress. Therefore, a biostimulant developed and standardized at CIBE, from the fermentation of plant waste, manure, and microorganisms [21] was applied to banana plants to determine the association of physiological effects and differential gene expression to elucidate molecular pathways activated in plants.

2. Results

2.1. Physiological Analyses

The application of the liquid biostimulant to the leaves showed a higher chlorophyll concentration in banana leaves than the control samples, especially after 48 h of application, even though this increment had no statistical difference in the timeline used (1 week top). The two-way ANOVA showed that the difference between treatments was statistically significant (p = 0.0314). Although the treated samples showed increased amounts of chlorophyll than the control, the increment was not statistically significant after the Tukey post hoc analysis (confidence level of 0.05) between the different time points (Figure 1A). On the other hand, a higher chlorophyll accumulation was observed in the biostimulant-treated plants. The changes in percentage of MSI were similar, showing unsignificant differences (p = 0.0911) among the control and the plants treated with the biostimulant (Figure 1B). The two-way ANOVA showed that the time points were a significant variable ($p = 2 \times 10^{16}$), with an increase in the membrane stability for both biostimulant and control treatments.

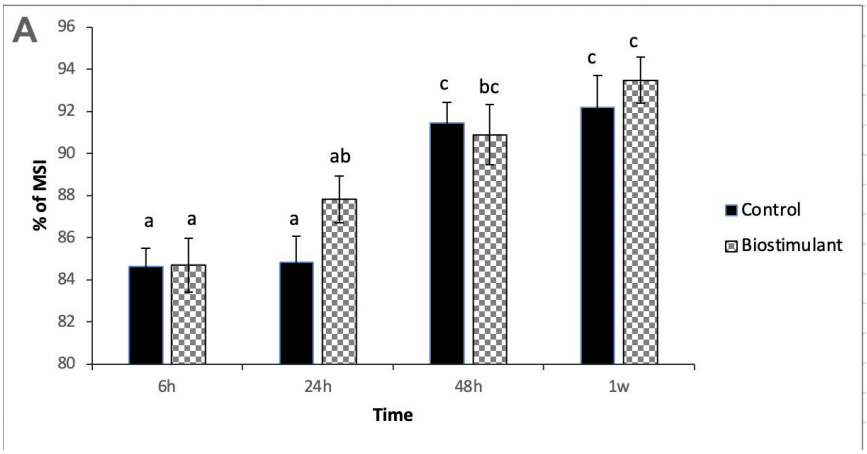

Figure 1. *Cont.*

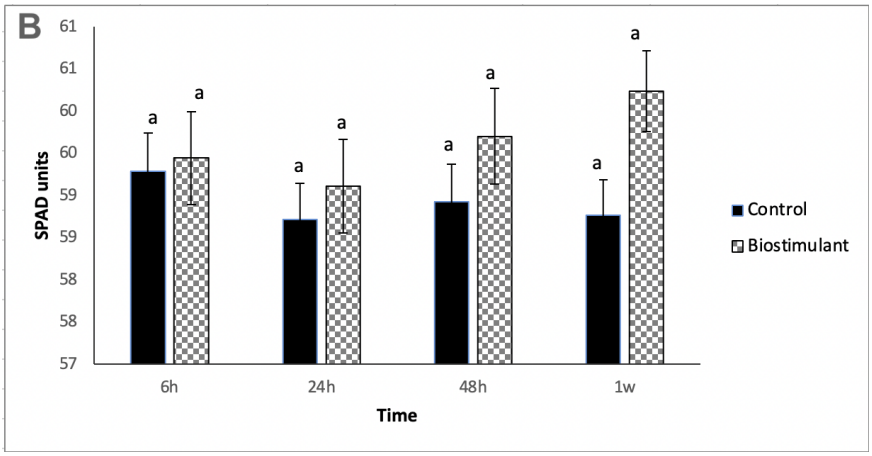

Figure 1. Chlorophyll Content and MSI percentage in banana leaves. (**A**) Chlorophyll content after foliar application of biostimulant and control (mock) treatment. (**B**) Percentage of MSI content after foliar application of biostimulant and control (mock) treatment. Letters a–c represents the statistics group differences between treatments.

2.2. Genes Identified in the Subtractive Library

A total of 270 sequences were obtained for the bioinformatics analysis, after processing and discarding redundant sequences (Data S1). Approximately 71% of these sequences, respectively, had homologous genes in other plant species according to a database analysis performed in the GenBank. The blast analysis indicated that most of the sequences belonged to the Musa genus (56%, Figure 2A), and approximately 4% of coincidence was encountered with bacterial organisms, represented mainly by *Pseudomonas fluorescens*. The plant sequences were used for mapping and annotation analysis by BLAS2Go software, where 54% and 46% obtained GO information for both analyses, respectively (Figure 2B).

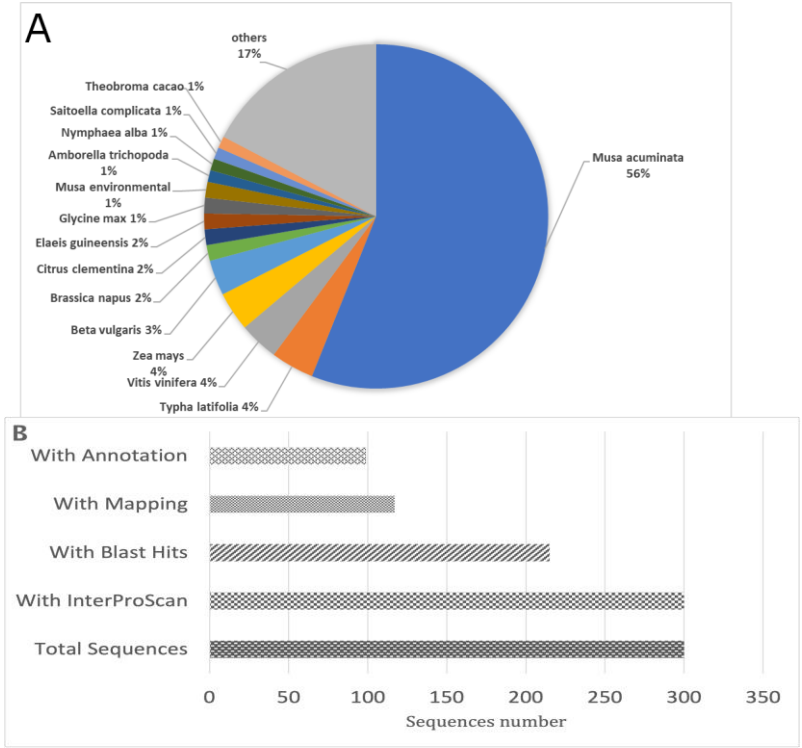

Figure 2. Statics Analysis of Sequences. (**A**) Top Hits BLAST of Representatives Species by BLAST2Go; (**B**) Statics a different level of analysis in the sequences by BLAST2GO.

Most of the sequences belonged to cellular compound, where the four main GO-terms were intracellular organelle, membrane-bounded organelle, intracellular, and intracellular part. Each category showed a total of 76 sequences in common. These sequences were highly represented by chloroplast compounds including the Ycf2 gene by 25%, RNA polymerase by 14.4%, and other genes (Figure 3). However, in other categories, such as biological process, most of the sequences belonged to the cellular metabolic process, with NADH gene predominantly with 30.5% and, for molecular function, the main GO-term was ion binding where GTP-binding nuclear Ran-2 contained most of the sequences (Figure 3).

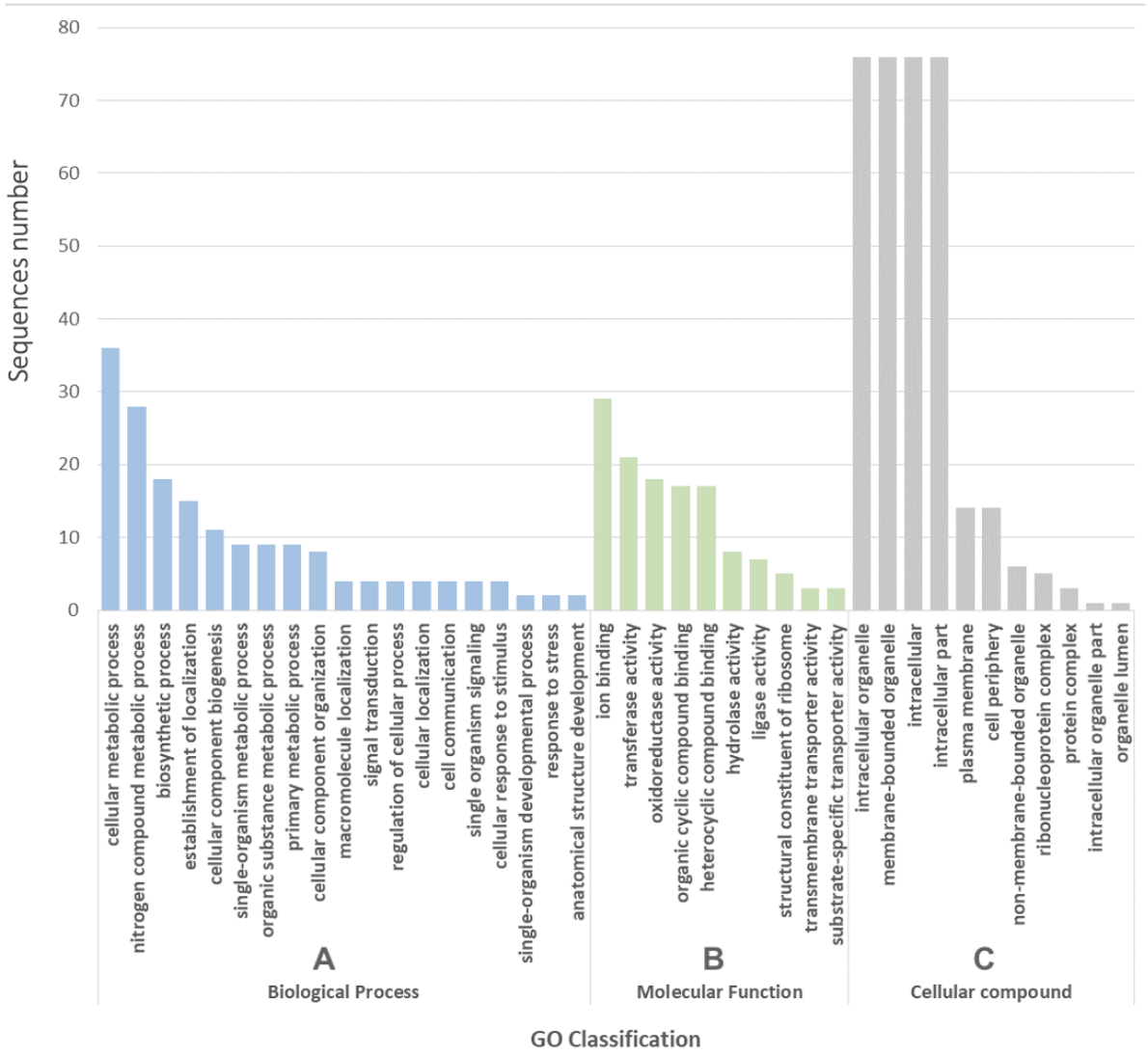

Figure 3. Categorization of ESTs by blast2Go level 2. (**A**) represents biological process; (**B**) represents cellular component; and (**C**) represents molecular function categorization.

2.3. Upregulated Genes after Biostimulant Application

For the analysis, sequences that belong to important conserved families, a super family, and domains were selected. Eight super-family proteins, followed by nine family proteins, and twenty-three domains, were encountered (Figure 4A–C). The main representatives for each family were ClpP/crotonase-like and LRR-like, NADH, Ycf2 and LTR, and finally ClpP/crotonase-like, Acetyl-coenzyme A carboxyltransferase, Acetyl-CoA carboxylase and NADH:quinone oxidoreductase/Mrp antiporter, respectively.

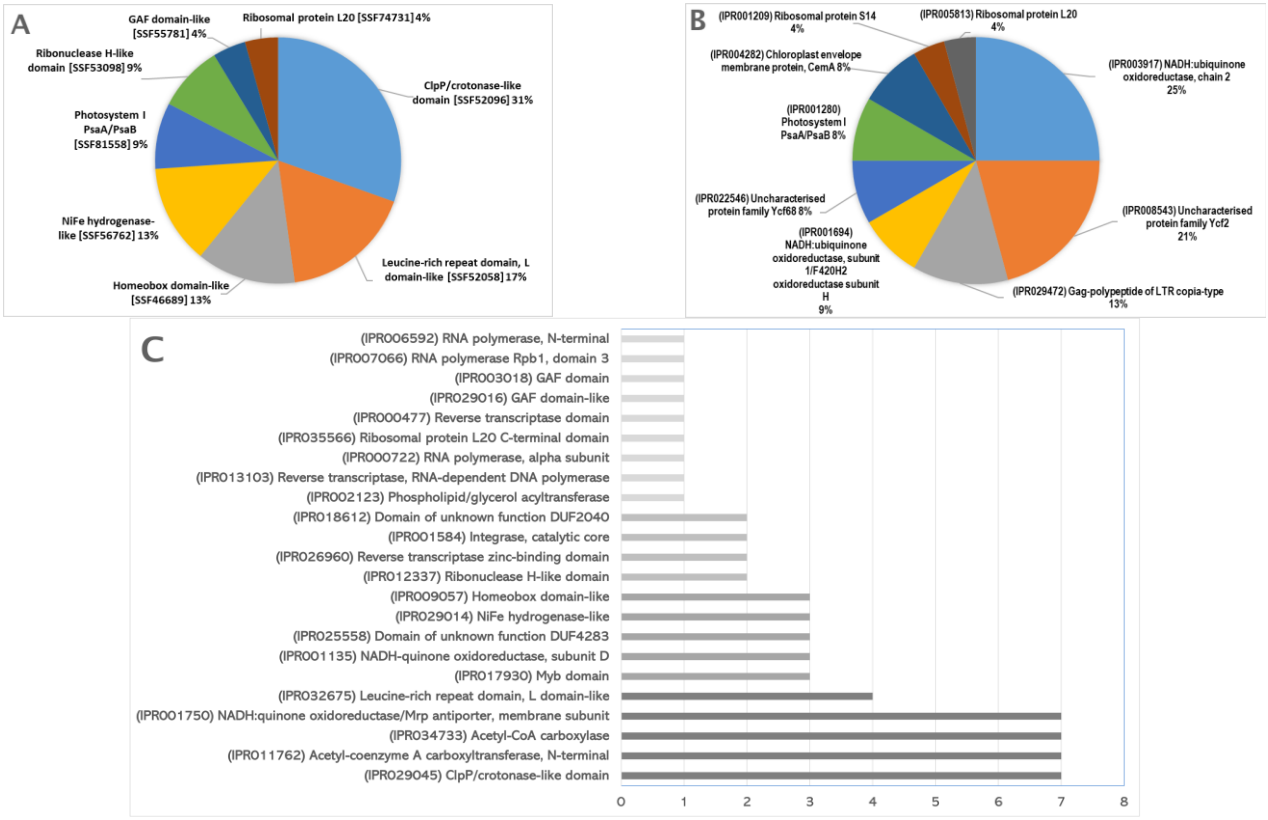

Figure 4. Pie Chart of Main Annotated Proteins and Domains found by BLAS2GO. (**A**) Super-Family Protein; (**B**) Family Protein; (**C**) Domains.

Five of the proteins that were found in this study belonged to five described pathways (Table 1), where the metabolism pathway of nucleotides was highly expressed by four enzymes, which were repetitive in 37% of the total sequences. Furthermore, transporters involved in the osmoregulation into photosynthesis such as a dehydrogenase (NADH) were encountered (Figure S1). With this result, we selected five genes which play a key role on plant fitness and pathogen interaction, according to the bioinformatics analysis on GO by using Blast2GO software, to analyze the expression in the plants. All of these genes had an expression variation after six hours of biostimulant application on the plant leaves. An increased expression after 24 h of application was observed in genes related to photosynthesis. On the other hand, the RNA catalyzer decreased the level of expression after the first six hours of biostimulant application. After 48 h, the overexpression was reduced almost until less than one-fold of the control used (Figure 5A).

Table 1. Description of activated pathways after biostimulant application.

Pathways	#Sequences	#Enzymes
Purine metabolism	21	3
Oxidative phosphorylation	18	2
Pyrimidine metabolism	16	1
Thiamine metabolism	5	1
Phenylpropanoid biosynthesis	2	1
Glutathione metabolism	2	1

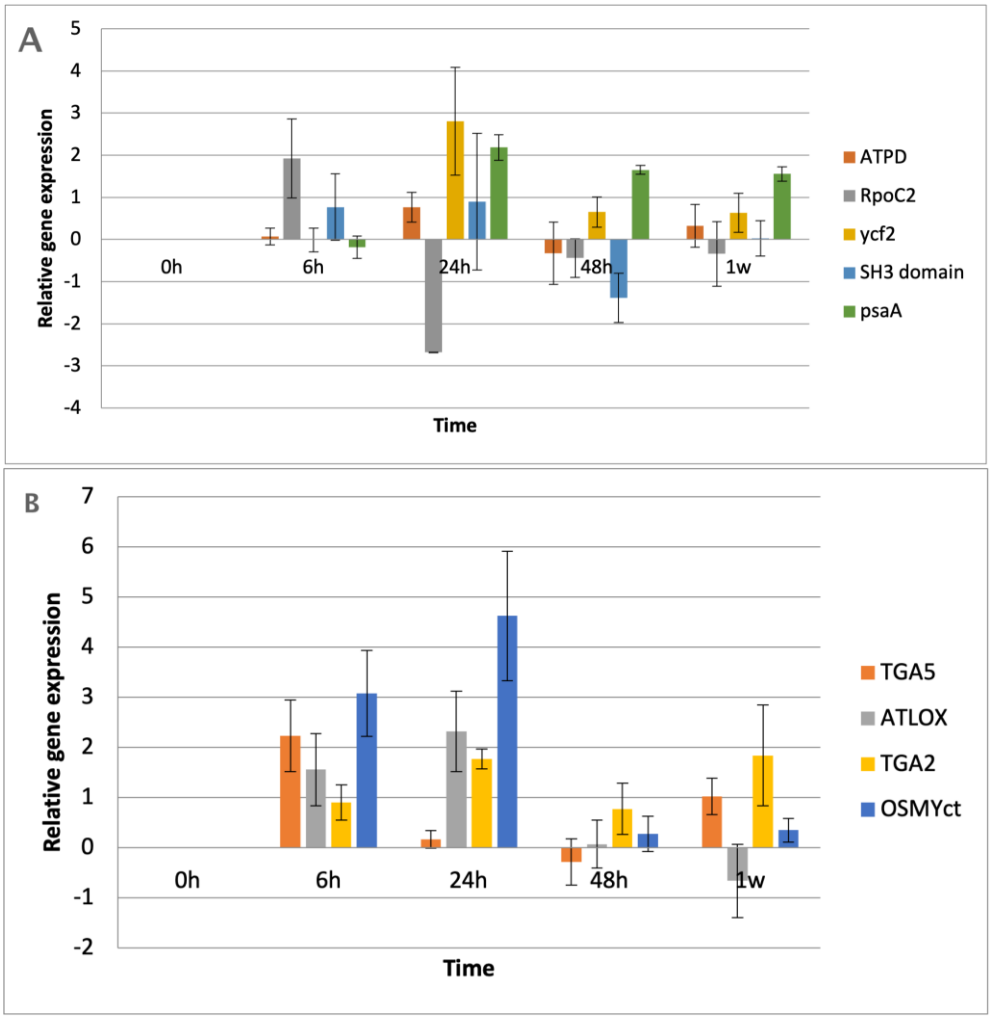

Figure 5. RT-qPCR Analysis of Differentially Expressed Genes after Biostimulant Application. (**A**) Selected genes related to photosynthesis and plant growth obtained by SSH library; (**B**) Genes related to SA and JA pathway activation.

To demonstrate that the biostimulant could induce plant resistance, the expression levels of markers involved in the SA *(TGA 2 and 5*) and the JA (*Myc2* and *Lox*) pathways were tested (Figure 5B). MaMyc2 showed a higher expression (3.075 fold and 4.62-fold expression) at 6 and 24 h after biostimulant application, respectively. A similar pattern but with lower expression was shown by the *MaLox*, 1.55- and 2.32-fold expression at 6 and 24 h after biostimulant application, respectively, and for both genes, the expression level was dramatically reduced after 48 h of biostimulant application. *MaTGA5* showed a 2.25-fold increase at 6 h after biostimulant application, but had a continuous reduction afterwards, showing a small increment one week after application. *MaTGA2* showed low expression levels at the beginning of the assay but, interestingly, showed an increased expression at 24 h and at one week after biostimulant application (1.76 and 1.84-fold, respectively).

3. Discussion

The study aimed to generate knowledge to understand the relation between the application of a standardized biostimulant and differential expression of discovered genes that could be translated into an improved performance in banana plants. The biostimulant proved to enhance plant growth, especially when it was used as a liquid solution, with a high concentration of microorganisms [29]. Biostimulants stands for an important field of study, to obtain benefit of living microorganisms which are present in soil plantations. The

use of biostimulants has been tested in some studies, showing a rate of nutrient uptake, increased tolerance against biotic and abiotic stress, enhanced crop production, or even soil health recovery [30,31]. As found in the present study, an increased gene expression of membrane related genes, photosynthesis genes, and osmoregulation related genes, such as NADH, may improve plant growth and stability under stress conditions. The physiological data demonstrated that biostimulant application did not impact negatively on the treated plants. Furthermore, the analysis of chlorophyll and MSI percentage was taken at early time points, which were not enough to see a statistical difference between treatments, but clearly showed an increase level of tendency on biostimulant treated plants. These results demonstrated that the biostimulant did not negatively affect the treated plants, by maintaining normal levels of chlorophyll content and percentage of MSI.

The subtracted library found that 4% of the obtained genes were associated with bacterial genomes, implying that some living bacteria remained in the biostimulant, suggesting that bacteria such as *Pseudomonas* spp. could establish within banana leaves. Therefore, this biostimulant could be used locally in crops, due to its association with beneficial microorganisms that may include different properties, such as phosphate solubility (*Bacillus*, *Aspergillus* and *Pseudomonas*) [32], with *Pseudomonas* spp. being the principal genus found in this study. Moreover, enhanced plant growth by microbial inoculants was associated with enhanced nutrient uptake and improved nutrient status of the plant. Treatments over a year, or even longer periods of time, could demonstrate better results for plant growth, where the changes in the plant would be more visible for biostimulant treatments over time [33]. Even though biostimulant preparation incorporates an initial addition of bacterial pool, this bacterial consortium, from initial development of the biostimulant to its application, is lacking identification of microorganism species dynamics.

The candidate genes identified in our study provided information about different molecular functions, cellular compounds, and biological processes that support the results of differential gene expression, mainly associated with biotic/abiotic stress and plant growth development. Relevant genes identified includes the *rpoC2* related to transcription in the chloroplast [34], suggesting an increase in gene transcription of photosynthesis-related genes. The *TRAB1* gene, which is related to the activation of the transcription factor *VP1* (which regulate the maturation and dormancy in plans seeds) by the induction of ABA-responsive elements (ABREs), was also increased [35]. Increased expressions of NADH electrons transporters [36], and psaA membrane proteins, located in photosystem II [37] were encountered, suggesting an increase in the photosynthetic capacity, which might be an indicator that the biostimulant used increased plant growth. Other studies using biostimulant-like mycorrhizas in plant roots have reported cellular transporter activation, which is important in amino acids, oligopeptides, and polysaccharides symbiosis processes. Furthermore, the genes identified proved to have an important role in the production of rich cysteine proteins in the root, to trigger a symbiotic interface [38,39].

Although the function is unknown for *ycf2*, the gene product might be involved in transcription regulations in the chloroplast, for genes of the nuclear-encoded RNA polymerases (NEP), which are involved in photosynthesis, sulfur and nitrogen reduction, and the biosynthesis of metabolites [40,41]. Therefore, an enhanced expression of *ycf2* could be related directly to plant growth and to an increase in photosynthesis after the biostimulant application. Nevertheless, this is one of the overexpressed genes related to the chloroplast, including *rpoC2* and *psaA* which are chloroplast proteins of the Plastid Encoded RNA Polymerase (PEP), involved in chloroplast biogenesis, associated with a *sigma factor gene 6* [42–46]. Furthermore, both NEP and PEP are involved in chloroplast transcriptional development, and the absence of those could result in a lack of photosynthesis [47,48]. Meanwhile, the SH3 domain (Src-homology 3) has been related to turgor changes in the cellular membrane associated with PBS2 and the protein SHO1 [49]. As part of the chloroplast transcription machinery, the increased expression of the *rpoC2* indicates that the photosynthesis in the plant increased. The application of biostimulants in plants showed regulation of gene expression and an increment of photosynthetic efficiency [50]. Therefore,

the increased expressions of genes related to photosynthesis are indicators of increase in photosynthetic efficiency. On the other hand, the *ATPD* gene is an important component of ATP synthases in chloroplast, suggesting that an increased expression might result in increased levels of ATP. Furthermore, ATP levels have been improved by biostimulants, when used as bioremedants in polluted soils [51]. Moreover, an increase in enhanced levels of proteins involved in ATP synthesis in mitochondrial and plastid-like ATP synthases could be associated with abiotic stress tolerance in genotypes plants [52].

To demonstrate the activation of defense mechanisms induced by biostimulant application, markers related to the jasmonic acid and salicylic acid hormones pathway were used. MaLox3 was found to be overexpressed after the application of the biostimulant. Lipoxygenases are known to play a key role in the JA, antimicrobial, and antifungal compounds synthesis [53,54]. An overexpression of a lipoxygenase (*CaLOX1*) in pepper was observed, resulting in enhanced salinity and drought tolerance in plants, which suggests that the application of the biostimulant might be an organic alternative to enhance stress tolerance in plants. Moreover, lipoxygenases have been involved in *Magnaporthe grisea* resistance in rice [55], which may suggest that the application of biostimulants could increase antifungal activity in banana plants, which may protect them from a potential *P. fijiensis* infection.

The transcription factor Myc2 is a basic helix loop helix (bHLH) protein that is known to be overexpressed after exogenous jasmonic acid application during herbivore attack and mechanical damage; furthermore, it has been recognized as the master regulator of the JA signaling pathway [56]. In *Nicotiana attenuata*, NaMyc2 has been related with the plant response to herbivore attacks and resistance, through the production of compounds such as phenolamides [57]. *Arabidopsis thaliana* root inoculation with *Pseudomonas fluorescens*, a beneficial plant bacterium, has been shown to downregulate *AtMyc2* repressors and enhance plant resistance to certain pathogens and insect herbivores, in a process called induced systemic resistance (SR) [58]. *OsMyc2* overexpression has shown to enhance abiotic stress tolerance in rice, thus in greenhouse conditions, transgenic plants showed better performance during longer periods of drought stress than the wild type controls [59]. Biostimulant treatments on banana plants enhanced the expression of *MaMyc2*, which may suggest that the plants would have enhanced tolerance against biotic and abiotic stress. Furthermore, *Myc2* expression is reduced at 48 h after biostimulant application, due to a self-feedback regulation of the *Myc2* transcription factor, as shown by Sanchez Timm [60].

Salicylic acid is a phenolic phytohormone, known to positively regulate the *NPR1* gene, which targets the *TGA* gene family to promote plant defense against biotrophic and hemibiotrophic pathogens [61]. Salicylic acid signaling has a negative crosstalk with jasmonic acid [62], which may explain a lower expression of *TGA* genes during the assay. TGA5, however, had a higher expression at 6 h after biostimulant application, which may be expected by a lower affinity with the *NPR1* in contrast to *TGA2*, as shown by Zhou et al. [61]. Certain herbivore insects seemed to exploit orally secret bacteria, such as pseudomonas, which lower JA-dependent plant resistance by increasing the SA plant signaling [63]. *TGA* expression levels seemed to recover at one week after application, as the *MaLox* and *MaMyc2* decrease. This change may be linked to bacterial recognition by the plant, as bacteria from the biostimulant could be found in the banana leaves after application [64]. In a plant–biostimulant–pathogen interaction, a SSH library was also performed and genes related to growth and pathogen resistance from banana plants were overexpressed [64]. Chávez et al. [64] discovered induced gene expression of genes related to growth and tolerance to (a)biotic stress. Furthermore, a blast analysis revealed genes from microorganism origin, suggesting that microorganisms present in the biostimulant are functional in the banana leaves.

Although du Jardín [19] clearly distinguished between a biostimulant and a biofertilizer, these types of products could not be clearly defined globally, resulting in constraints regarding regulation, patent filling, and application [65]. Current regulation worldwide about biostimulants is not clearly defined, being complex and diversified [66]. Therefore, the registration of biostimulants or biofertilizers is often complex in most parts of the

world [67]. In Ecuador, the governmental entity AGROCALIDAD is responsible for the registration, authorization, and control of any agricultural input. According to the Technical Manual for the Registration and Control of Fertilizers, Soil Amenders and Related Products for Agricultural Use (issued on the 21 February 2020 (www.agrocalidad.gob.ec/wp-content/uploads/2020/05/ac6.pdf; accessed on 13 January 2023), "biol" is considered to be any product resulting from the anaerobic (fermentation) decomposition of materials derived from animals (especially manure) and vegetables (crop waste) which contain nutrients that are easily consumed by plants. This could lead to more vigorous and resistant plants. "Biols" often contain phytohormones; therefore, they are considered biostimulants. In China, a definition of a biostimulant is not clearly stated. The Agricultural Standard of China NY/T 3831-2021, which was published in November 2021, indicates that organic water-soluble fertilizers contain biostimulants (www.cirs-group.com/files/aty8b0q8or9c/content/2022/02/bd2jccn5n30g.pdf, www.biostimulant.com/regulatory/#:~:text=The%20harmonized%20definition%20of%20a,b)%20tolerance%20to%20abiotic%20stress; accessed on 13 January 2023).

4. Materials and Methods

4.1. Plant Conditions

A total of 54 banana plants derived from in vitro culture were used in the bioassay. Plants were acquired commercially from the "Agripac" company. Plants in bags with substrate were maintained in the greenhouse for three weeks. Six replicates were performed in controlled conditions in the greenhouse: (i) application of biostimulant at 30% concentration (diluted in water) to the third unfolded leaf of the banana plant, and (ii) application of sterile water as a control (mock application). Leaf material was taken in the following time points: 0, 6, 24, 48 h and 1 week after biostimulant application and stored at −80 °C until processing.

4.2. Biostimulant Conditions

The liquid biostimulant was elaborated at CIBE-ESPOL, in the Agricultural Technics Area, according to Jiménez et al. [21]. Briefly, raw materials from organic farms were used for the fermentation process. Materials used included fresh cattle manure (40 kg), sugarcane molasses (four liters), soybean leaves (10 kg), burned rice husks (10 kg), and eight liters of native microorganism. These materials were mixed with water in a 200 L tank for fermentation in anaerobic conditions. The native microorganisms were collected from organic banana farms and were multiplied according to Jiménez et al. [21]. Briefly, growth medium was prepared using 150 g of boiled rice, 400 mL of a mixture containing: 40 L sugarcane molasses, 27 kg fish meal, 680 g NaCl and 80 L of water. The mixture was boiled, placed in small containers covered by a mosquito net, and placed near trees in an organic banana farm. Twenty-one days later, the mixture with unidentified microorganisms was transferred to a 500 L tank with the same proportion of sugarcane molasses-fish meal-NaCL-water as indicated above. After 7 days, the mixture of native microorganisms was used for the preparation of the biostimulant. The parameters of the biostimulant include pH: 3.81–4.17, conductivity: 20.23–24.47 mS/lcm, solutes: 5.69–7.81 g/L, density: 1.03–1.04 g/cm^3, organics remains: 1.89–4.91%, and salinity: 8.95–10.59 ppm, at a temperature of 26.6 °C.

4.3. Physiological Parameters Analyzed

Membrane stability index (MSI) was determined following the protocol established by Sairam et al. [68], using the leaves of each plant under treatment at each time point of the assay (6h, 24h, 48h, and 1 week after biostimulant application). The conductivity was taken using the conductivity meter 441 (109122-1 Rev. A, 10/96; Corning, Woburn, MA USA 01801). Chlorophyll content of each treatment was performed by using a Chlorophyll meter Konica Minolta SPAD 502. Parameters were taken at 6, 24, 48 h, and 1 week after biostimulant application. The statistical analysis of the MSI and Chlorophyll content was analyzed by Excel version 2013 (Redmond, Washington, DC, USA) and Rstudio platform,

version 2016 (Boston, MA, USA) [69]. A two-way ANOVA was applied to find the statistical significance between treatments, and Tukey's test (0.05 significance level) was used for comparing and grouping all the treatments.

4.4. Total RNA Isolation and cDNA Synthesis

Plant tissue was collected at 0, 6, 24, 48 h, and 1 week after application for each condition. Collected samples were immediately submerged in liquid nitrogen and stored at −80 °C until isolation. RNA isolation was performed by a phenol chloroform-isoamyl alcohol (phenol-chloroform-IA 24:24:1concentration, Sigma Aldrich, St. Louis, MO 68178, USA) protocol. Briefly, a lysis buffer containing NaCl (400 mM), Tris-HCl (10 mM, pH8), EDTA (2 mM, pH8), 2% of polyvinylpyrrolidone (360,000) and β-mercaptoethanol at 0.7% was used. A two-step precipitation with phenol-chloroform-IA was performed following precipitation with a chloroform-IA (24:1) mix (Sigma Aldrich, St. Louis, MO 68178, USA). Precipitation of RNA was performed using sodium acetate (3M) leaving the samples overnight at −80 °C. Finally, two washes with 70% ethanol were performed, and total RNA was resuspended in 50 µL of sterilized and piethyl pyrocarbonate (DEPC)-treated water. The isolated RNA was stored at −80 °C until cDNA synthesis. Synthesis of cDNA for library generation was performed from a pool of samples applied with the biostimulant (tester) and control samples (driver). The pool was generated by using three replicates of each time point collection (0, 6, 24, 48 h, and 1 week after biostimulant application) for each treatment, in a final concentration of 20 µg of RNA for each pool (tester and driver). The synthesis was generated using SuperScript® Double-Stranded cDNA Synthesis Kit (Invitrogen, Waltham, MA 02451, USA) following the fabricant instructions. The cDNA synthesis for the differential expression analysis by RT-qPCR was performed using SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Waltham, MA 02451, USA), using 2 µg of total RNA isolated previously and according to the manufacturer.

4.5. Library Generation and Analysis

The genetic library was developed with the Subtractive Suppression Hybridization technique, using the PCR-Select™ cDNA Subtraction Kit (Clontech Laboratories, Inc., Mountain View, CA, USA) and following the manufacturer's protocol, except for the cDNA synthesis, which was generated as described above. The subtractive sequences were cloned into the pGEM®-T Easy Vector System (Promega, Madison, WI, USA) and transferred into Escherichia coli competent cells. Sequence insertions were determined by standard PCR (M13 primers forward seq: GTAAAACGACGGCCAG and reverse seq: CAGGAAACAGC-TATGAC). An alkaline lysis protocol was performed for plasmid isolation, using 3–5 mL of bacterial growth into Luria-Bertani (LB) broth medium with the corresponding antibiotic. Briefly, cells were collected by centrifugation and resuspended in a solution containing 50 mM glucose, 25 mM Tris-HCl at pH 8.0, 10 mM EDTA, and RNase A. Samples were incubated in ice for 5 min, and a second solution with 0.2N of NaOH, 1% of SDS was added. Tubes were mixed by inversion and incubated in ice for five minutes. A third solution was added, containing glacial acetic acid and 5 M of potassium acetate at pH 5.5, and samples were incubated for 5 min in ice. Centrifugation at 13,000 RPM for 5 min was performed and the supernatant was transferred into a new tube with isopropanol in a ratio of 1:1. Finally, samples were centrifuged at 13,000 RPM for 10 min, the pellet was washed with 70% ethanol, and suspended in TE pH 8.0 [70]. Sequencing of plasmids containing the ESTs was performed commercially. Sequences were analyzed in the GenBank database (BLASTn and BLASTx). To determine activated pathways in plant, sequences with annotated proteins were used for further analysis, starting with categorization of orthologous genes of all the sequences at three levels: (i) biological process, (ii) cellular compound, and (iii) molecular functions generated by BLAST2GO software [71]. This software allows us to understand and infer possible genes in our organisms target by using annotated sequences in other organisms (gene ontology, GO; accessed 13 January 2023) [71]. Selected ESTs were analyzed for similarities in the banana genome [72].

4.6. Pathway Activation and Gene Expression Analysis

For normalizing, the elongation factor gene (EF1-F2: CGGAGCGTGAAAGAGGAAT, EF1-R2: ACCAGCTTCAAAACCACCAG) was used as an internal reference gene according to Sánchez Timm et al. [61]. Five selected genes from the library and four genes from pathways related to inducing a response in plants were used in the analysis by using the primers indicated (Table 1). The primers were designed by primer3 software (https://primer3.ut.ee/; accessed on 13 January 2023).

RT-qPCR was performed using GoTaq® qPCR Master Mix (Cat# A6002, Promega, Madison, WI, USA), using 10 µL of GoTaq® qPCR, 1 µL of each primer (1 µM final concentration) per reaction and 2 µL of cDNA (1:5 diluted) in a total of 20 µL per reaction for RT-qPCR. The thermal cycler protocol used had a Hot-Start Activation condition at 95 °C for 2 min, following 40 cycles of denaturation at 95 °C for 15 s and annealing/extension 60 °C for 60 s, then one cycle of dissociation at 68 °C for 20 s, and finally a dissociation step at 95 °C for 15 s, following a melting curve generation. The normalization for data analysis was performed using the 2–ΔΔCT method [73]. All statistical analyses were performed using the Excel 2013 and RStudio version 2016 [69].

5. Conclusions

A liquid biostimulant was tested to examine its physiological effect and for the identification of differential-expressed genes in banana plants. The physiological analysis of biostimulant-treated plants demonstrated no negative effect. The liquid biostimulant increased the membrane stability index (MSI), which is directly related to a higher CO_2 exchange and assimilation rate. Additionally, the total chlorophyll content increased in plants after biostimulant application, suggesting a significant enhancement of the photosynthesis ability of the plants. This study generated new information about the interactions between biostimulant application and plant development at a molecular level, due to the generation of a SSH library for the identification of genes expressed after biostimulant application. Although the bioassays were not designed to induce stress in the banana plants, biostimulant application boosted the expression of some biotic and abiotic stress related genes, suggesting that biostimulants could interact in the activation of some pathways related to stress tolerance in the plant. The subtracted EST library also contained genes involved in plant respiratory mechanisms and photosystem association. The gene expression analysis corroborated the overexpression of the selected genes ycf2, rpoC2, and psaA, which belong to chloroplast plastid, playing an important role in plant development. Those genes are strongly associated with plant growth in the photosynthesis system and have proved to be induced by biostimulant application. These results suggest that the biostimulant could enhance the growth in plants, increasing the photosynthesis system.

Biostimulant application immediately enhance the JA signaling pathway, which may suggest that treated plants may show increased tolerance against certain kind of stress. Further assays are needed to corroborate this. Even though SA signaling was negatively affected by the SA-JA crosstalk, an increase in TGA2 expression was observed one week after the biostimulant application treatment, which may be related to the plant recognition of the microorganisms present in the biostimulant. Thus, these findings suggest that the liquid biostimulant may promote an induced systemic resistance effect in banana plants, which can be beneficial as an organic alternative to control certain types of pathogens, such as *Pseudocercospora fijiensis*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13020415/s1, Data S1: DNA sequences generated from the SSH library; Figure S1: Oxidative phosphorylation.

Author Contributions: T.C.-N. and R.P.-C. were involved in the elaboration of experimental tests in greenhouse, physiological data recollection, and RNA isolation. T.C.-N. and N.B. were involved in SSH library generation, and also involved with L.S.-T. and E.S.-O. in data analysis including interpretation of sequences. R.P.-C. and T.C.-N. drafted the manuscript. L.S.-T., E.S.-O. and N.B.

were involved in revision for critically important intellectual content. E.S.-O., L.S.-T. and N.B. conceived and designed the experiments. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: DNA sequences are available as Data S1.

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