Determination of Molecular Interactions Between Fraser's Photinia (Photinia X Fraseri) and Its Endophytic Bacterium PGB_Invit

Nil TÜRKÖLMEZ

Gebze Technical University: Gebze Teknik Universitesi

Merve KARAKAYA

Gebze Technical University: Gebze Teknik Universitesi

Hamit Ekinci

Gebze Technical University: Gebze Teknik Universitesi

Stuart J. LUCAS

Sabancı University: Sabancı Universitesi

Özlem AKKAYA

Gebze Technical University: Gebze Teknik Universitesi

Mine Gül Şeker

Gebze Technical University: Gebze Teknik Universitesi

Ceyhan KAYIHAN

Başkent Üniversitesi: Baskent Universitesi

Yelda Ozden (✉ yelda75@yahoo.com)

Gebze Teknik Universitesi <https://orcid.org/0000-0002-9799-3648>

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Abstract

An endophytic beneficial bacterium, was isolated and characterized from *in vitro* grown microshoots of Fraser's Photinia in our previous studies. This bacterium, nominated as PGB_invit (*Pseudorhodoplanes* sp.), has ability to fix nitrogen and produce plant growth promoting regulators such as indole acetic acid and gibberellic acid. Probably because of all of these features, this specific bacterium promotes *in vitro* proliferation of Fraser's Photinia microshoots and enables microshoots to be conserved at 25 °C for up to 16 months without requiring periodic subculture. Due to its beneficial influence on growth of Fraser's Photinia, it is aimed to identify physiological, biochemical and molecular influences of this bacterium together with determination of its the localization in plant tissues. Our results showed that inoculation with PGB_invit bacteria (10⁶ cfu/ml) has increased chlorophyll content without causing any severe oxidative stress. Moreover, the genes [gene bank ID XP_008352422.1 (predicted "MID1-Complementing Activity 1"), PWZ54828.1 (Ubiquitin conjugating enzyme E2), XP_028948963.1 (predicted "E3 ubiquitin-protein ligase RHA1B-like"), XP_008339965.2 (uncharacterized protein), XP_028948163.1 (4-coumarate-CoA ligase-like 9), and XP_028953023 (methionine aminopeptidase 2B-like)] which are upregulated or downregulated after inoculation of this bacterium according to RNA-seq analysis, were also confirmed with qRT-PCR in order to understand its molecular interaction with its host.

1. Introduction

Plant-microbe interactions such as competition, commensalism, mutualism, and parasitism are a vital part of our terrestrial ecosystem (Wu et al., 2009). Hence, a wide variety of microorganisms such as bacteria, fungi, actinomycetes, algae, and protozoa can be found in soil (Kumar, 2016) and a great deal of research has been performed on Plant Growth-Promoting Rhizobacteria (PGPR) among them.

PGPRs are non-pathogenic for plants and strongly increase the yield of the plant with their beneficial effects by colonizing on the surface or in internal tissues of the plant roots (Babalola, 2010; Kargapolova et al., 2020). Among PGPRs, endophytic bacteria, which are colonize internal tissues, communicate and interact with the plant more efficiently than rhizospheric bacteria and in a result of this, their beneficial effect will be more efficient (Hernández-Soberano et al., 2020). These effects can happen through one or more mechanisms such as production of phytohormones, metabolites and siderophores, fixation of nitrogen, biocontrol of root zone pathogens, uptake of nutrients or stimulation of plants to produce these molecules. Many endophytic strains can also regulate plant immunity responses and developmental processes such as cell division, cell elongation, cell differentiation by producing and/or degrading a kind of auxin, i.e., indole acetic acid (IAA) (Sturz et al., 2000; Khan et al., 2020).

As a result of all these features, they are considered as a way to reduce the use of chemical fertilizers for plant growth (Wu et al., 2009). It is well known that excessive use of chemical fertilizers not only deteriorates soil quality and fertility, but also it negatively affects fertile lands that can be used for agriculture (Chauhan et al., 2022). For this reason, interest in studies on this subject has increased in recent years. For instance, Llorente and Larraburu (Llorente and Larraburu, 2013) inoculated Fraser's Photinia with *Azospirillum brasilense* and observed early rooting of Fraser's Photinia shoots and significant increase in root fresh and dry weights, root surface area, and shoot fresh and dry weights in comparison with controls.

In 2019, Chen and Liu, inoculated soil with *Pantoea* sp. S32 (phosphate-solubilizing bacterium) and they found that this bacterium converts insoluble forms of phosphorus (P) to accessible forms. In addition to this, they performed a rice seedling experiment with this bacteria at the same time and recorded that there is a significant increase in height, biomass, root growth, and P uptake of rice (Chen and Liu, 2019). Moreover, Adesemoye et al. (2009), reported that, with the use of PGPR strains such as *Bacillus amyloliquefaciens* IN937a and *Bacillus pumilis* T4, plant growth, crop yield and flow of nutrients were increased using 75% of the recommended fertilizer rate. Therefore, it is possible to use PGPRs as biofertilizers in agriculture (Rosenblueth and Martínez-Romero, 2006).

Besides their usage as microbial fertilizers, it is also vital to understand PGPR-plant molecular interactions to reveal out how this beneficial interaction work well in these organisms. Approaches such as transcriptomics (RNA-seq and microarrays etc.) are known as powerful tools to understand bacterial gene functions in the plant microbiome (Nobori et al., 2021). RNA-seq transcriptional profiling, which allows detection of differentially expressed genes involved in the oxidative stress response, plant immunity, production of plant hormones and secondary metabolites, has been utilized in the literature to detect differentially expressed genes profiles of different plant species inoculated with rhizobial strains. Accordingly, distinct transcriptomic responses were evidenced in soybean (*Glycine max*) after inoculation with rhizobial strains belonging to two different genus, *Bradyrhizobium japonicum* and *Sinorhizobium* (Ensifer) *fredii* (Yuan et al., 2016). Another study compared transcriptomic profiles of *Lotus japonicus* upon inoculation with compatible rhizobial, non-adapted rhizobial and pathogenic bacterial strains, and revealed that compatible rhizobia did not induce early defense-like responses (Kelly et al., 2018).

In our previous reports, the presence of a unique bacterium, that positively affects plant growth, has been identified during tissue culture studies with Fraser's Photinia (Gül Şeker et al., 2017; Şah et al., 2019). According to phylogenetic analysis, this putatively endophytic bacteria, PGB_invit, is also a PGPR from the Alphaproteobacteria group that has capability to fix nitrogen and produce some plant growth promoting regulators such as indole acetic acid (IAA) and gibberellic acid (GA₃). The most similar reported species was found to be *Pseudorhodoplanes sinuspersici* strain C16-RIPI 110 (JX500274.3), isolated from oil contaminated soils in Siri island, Iran, with 16S r-DNA analysis (Tirandaz et al., 2015). Although, there are some similarities like being rod-shaped, staining Gram-negative, being facultatively anaerobic, occurring as small colonies on agar plates, having catalase production and growing optimally 30 °C and pH 7, they have differences in fatty acid features and carbon sources that they utilize. Moreover, it also determined that β-polyhydroxybutyrate production and intracellular polyphosphate accumulation occurred in PGB_invit cells. Thus, PGB_invit could be considered as a putatively new species of *Pseudorhodoplanes* sp.

It has been previously shown that PGB_invit promotes *in vitro* proliferation of Fraser's Photinia without subculturing for up to 16 months, as well as rooting and acclimatization of *in vitro* propagated shoots (Şah et al., 2019). However, there are still unsolved questions on, for instance, the optimum bacterial inoculation concentration, incubation time and its location in plants. Moreover, the physiological, biochemical and molecular influences of this bacterium on its host are still unknown to understand the mechanism of this positive interaction. Hence, it is aimed to optimize the beneficial effects of the PGB_invit on

Fraser's Photinia under *in vitro* and subsequently *in vivo* conditions, by performing physiological and biochemical determinations. In addition, molecular interactions between PGB_invit and Fraser's Photinia in *in vitro* conditions is also investigated by analyzing the changes in gene expression levels with RNA-seq and qRT-PCR analysis.

2. Material & Methods

2.1. PGB_invit Culture

Mineral-Peptone-Yeast-Extract (MPYE) medium (Davidson et al., 1989) which contains peptone (3g/L), yeast extract (3g/L), 1M MgCl₂ (1 ml) and 1M CaCl₂ (1 ml) with pH:7.0 were used to promote the growth of PGB_invit at 30°C. Bacterial culture initially started from -20°C stocks into MPYE broth and bacteria streaked to MPYE agar by sterile loop after 7 days of growth. Following the growth for 7 days in MPYE medium, colonies became apparent and a single colony from this medium inoculated into MPYE broth again and incubated there for 7 more days at 30°C. This bacteria culture used to inoculate Fraser's Photinia's *in vitro* tissue culture. Following the adjustment of the turbidity to 0.6 McFarland (approximately 10⁸ cfu/ml), three different serial dilutions (10², 10⁴ and 10⁶ cfu/ml) were prepared to find the optimum inoculation concentration.

2.2. *In vitro* proliferation of Fraser's Photinia

Shoots of Fraser's Photinia were provided from Gebze Technical University garden. After surface sterilization of shoots by washing under water for 90 min., 70% v/v EtOH for 10-15 seconds, rinsing with 1.5% v/v bleach (NaOCl) for 20 minutes, washing 3 times for 10 minutes with sterile distilled water, shoot tips were excised and transferred to fresh QL medium (Quoirin and Lepoivre, 1977) containing 1 mg/L BA (proliferation medium). Cultures were incubated in a growth room at 25°C under a 16 h photoperiod of 36 µmol m⁻² s⁻¹ photosynthetic photon flux provided by cool-white fluorescent lamps. Following their proliferation for four weeks, microshoots subcultured monthly (at least for 3 months) to proliferation medium and then excised shoot tips were used in the inoculation experiments.

2.3. Determination of the optimum inoculum for PGB_invit

The turbidity of PGB_invit bacterium in fresh culture medium was adjusted to 0.6 McFarland at OD₆₀₀ in physiological saline and from that, three different serial dilutions (10², 10⁴ and 10⁶ cfu/ml) were prepared. These dilutions were spread on QL medium by Drigalski spatula (100 µl for each). Non-PGB_invit-treated shoot tips served as a control group and they were inoculated only with physiological saline solution (PSS) to reveal out its possible effect on growth of plants. Moreover, three groups were prepared by inoculation of plants with bacterium in three concentration (as mentioned above) which are inactivated in 95 °C for 20 minutes.

All groups of plants (8 groups as total) were cultured in *in vitro* proliferation conditions explained before in different time periods, 30, 45 and 60 days without subculturing. Subculturing was not carried out in those trails as we previously reported that PGB_invit has capability to support *in vitro* shoot growth in Fraser's Photinia without subculturing up to 36 months (Şah et al., 2017). The results are evaluated as proliferation percentage (%), shoot/explant rate and shoot length in all plant groups. According to the results, the optimum inoculation concentration and the optimum *in vitro* proliferation time period determined as the group of inoculated with 10⁶ cfu/ml active PGB_invit bacteria and 60 days for best development of plants. Therefore, we performed our experiments further to understand the effects of plant-bacteria interaction on physiological, biochemical and molecular levels, on shoot growth together with rooting and acclimatization.

2.4. Determination of PGB_invit's Effect On Amount of Photosynthetic Pigments

PGB_invit's effect on photosynthetic pigments concentration (chlorophyll a, chlorophyll b, carotenoids) were determined according to Lichtenthaler (1987). Quantities of pigments were calculated according to the following equations and the result was evaluated as µg/ml/g FW.

Chlorophyll a (Chl a) = (11.24 x A_{661.6}) - (2.04 x A_{644.8})

Chlorophyll b (Chl b) = (20.13 x A_{644.8}) - (4.19 x A_{661.6})

Carotenoid (Cx+c) = [(1000 x A₄₇₀) - (1.9 x Chl a) - (63.14 x Chl b)]/214

2.5. Determination of antioxidant enzyme activities

To reveal the effect of the PGB_invit-plant interaction on the activities of antioxidant enzymes, plant samples were taken in 1st, 3rd and 7th days after inoculation with PGB_invit on QL medium plates. Control group samples were taken in the same days after they subcultured on QL medium plates. 0.2 g plant samples were homogenized in 1 ml suspension buffer [(50 mM potassium phosphate buffer (pH 7.8) containing 1 mM ethylenediamine tetraacetic acid (EDTA) and 2% polyvinyl pyrrolidone (PVP)] with mortar and pestle. Then, homogenates were centrifuged at 13000 g for 30 minutes at 4°C and the supernatants were transferred into new tubes on ice. Total soluble protein content was determined following to Bradford (1976) method. Catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and guaiacol peroxidase (POX) activities were determined according to the Chance and Maehly (1955), Wang et al. (1991), Sgherri et al. (1994) and Quesada et al. (1990), respectively. All activity experiments were performed as 3 biological and 2 technical replicates.

2.6. Determination of Proline, Malondialdehyde (MDA) and Hydrogen peroxide (H₂O₂) contents

MDA and H₂O₂ contents are evaluated following the 1st, 3rd and 7th days of inoculation of plants with PGB_invit on QL medium. Control group samples were also evaluated at the same days after subcultured on QL medium plates. The proline content was determined according to Bates et al. (1973) while MDA and H₂O₂ contents of plant samples were determined according to Heath and Packer (1968), and Sergiev et al. (1997), respectively. All the measurements were done with 3 biological and 2 technical replicates.

2.7. Determination the effect of PGB_invit on rooting and *in vivo* acclimatization of Fraser's Photinia

Following optimum *in vitro* culture without subculturing, microshoots that are inoculated with optimum PGB_invit concentration transferred to semi-solid QL medium with 0.5 mg/l indole butyric acid (IBA) and without any auxin application for their rooting. The microshoots which are not inoculated with bacterium (control group) were also taken to rooting applications. Microshoots with at least 0.2 cm roots were accepted as they rooted. The rooting percentages (%) of microshoots, the number of roots per microshoots and average root lengths (cm) were calculated after they incubated for 4 weeks in a growth room at 25°C under a 16 h photoperiod of 36 µmol m⁻² s⁻¹ photosynthetic photon flux provided by cool-white fluorescent lamps. Control and PGB_invit inoculated plants which are rooted *in vitro* conditions were taken from medium and washed under tap water and then they were placed into small pots containing a mixture of sterile soil, peat and perlite for their acclimatization in greenhouse conditions. The first 15 days of transfer, the pots were covered with stretch film for adaptation to low humidity conditions and a hole was gradually opened on the stretch film every day. The plants were irrigated at regular intervals. After 15 days, the stretch films were completely removed and the plantlets continued to grow in greenhouse conditions. At the end of 30 days, the acclimatization percentages (%) of control and inoculated plants were calculated. At least 30 microshoots were used for rooting and acclimatization experiments and repeated twice.

2.8. Screening the location of the PGB_invit in plants

2.8.1. GFP-Plasmid Transformation

The bacteria were transformed with a Green Fluorescent Protein (GFP) plasmid (pSEVA227-G) in order to determine the location of PGB_invit. The donor strain *Escherichia coli* (*E. coli*) DH5α carrying pSEVA227-G [provided by Victor de Lorenzo Lab, (<http://seva.cnb.csic.es/>)] to be introduced into PGB_invit strain via conjugation as described in Martínez-García and de Lorenzo (2011). For this aim, the donor strain *E. coli* DH5α containing pSEVA227-G and *E. coli* HB101 strain containing the mating helper plasmid pRK600 were grown in LB broth, 50 µg/ml Kanamycin (Km), and 30 µg/ml Chloramphenicol (Cm), respectively. Then the cells were washed twice with 10 mM MgSO₄ solution and each bacterial suspension was mixed in a 1:1:1 ratio. in 5 ml of 10 mM MgSO₄. These mixtures were placed on LB Petri plates in different proportions and allowed to grow at 30°C. After at least one day, LB Petri plates were washed with 5 ml 10 mM MgSO₄ and the cells at appropriate dilutions were spread over the selective medium (MPYE medium containing 50 µg/ml Km). After the Petri plates were incubated at 30°C for 7 days, GFP-positive colonies were initially selected using the imaging system (Azure Biosystems, C600). Then positive transconjugants were tested by PCR amplification using (5'-GCGGCTGCAGGCATGCAGGAGGAAAAACATATGAGTAAAGG-3'; 5'-GCGGAAGCTTCTATTGTATAGTTCATCCATGCC-3') primers to confirm the presence of the ~700 bp gfp fragment. The sequence was amplified using Go Taq DNA polymerase (Promega, Madison, WI) and the PCR conditions are: predenaturation at 95°C for 2 minutes, 30 cycles with denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 1 minute, and with a final extension at 72°C for 10 minutes.

2.8.2. Inoculation of *in vitro* Fraser's Photinia microshoots with GFP-labeled PGB_invit

PGB_invit strain labelled with GFP was grown in MPYE broth (with kanamycin) and density of the bacterial culture was adjusted to 0.6 McFarland with a turbidity-meter. The Fraser's Photinia microshoots those grown in *in vitro* tissue culture conditions for 60 days were inoculated with the PGB_invit culture in MPYE broth by following steps. 2 ml bacterial culture (0.6 McFarland) was spread over the entire ½ MS0 medium (Murashige and Skoog, 1962) of plant tissue culture vessels by Drigalski spatula. After that, shoot and leaf parts of microshoots were cut in a laminar flow cabinet with sterile lancet and placed on the vessels. In addition, to make sure that the inoculation of plant with bacteria, the leaves and shoots were first kept in the bacterial culture for about 10 minutes before they placed on ½ MS0 mediums. To be used as a control group, some shoot and leaf parts were placed on the plant tissue culture vessels containing only ½ MS0 medium without bacteria. All the plants were incubated at plant growth chamber for 5 days. 2 µl propidium iodide (PI) was used to stain plant tissues and incubated in dark at room temperature for 20 minutes. Visualition of stained cells were imaged in Zeiss LSM880 Airyscan confocal microscope (Carl Zeiss, Jena, Germany). GFP is stimulated at 488 nm and the emitted light is gathered at 510-560 nm and becomes visible (Chi et al., 2005). For this purpose, PI was stimulated with wavelength of 488 nm and a measurement was taken at 565 nm wavelength. For GFP, it was stimulated with 488 nm laser and a measurement was taken at 530 nm wavelength. 10X and 20X objective lenses were used throughout the study.

2.9. Determination of PGB_invit-Fraser's Photinia Interactions at the Molecular Level

2.9.1. RNA isolation

After 60 days incubation in *in vitro* conditions, Fraser's Photinia microshoots that were inoculated with active and inactive PGB_invit, and the non-inoculated control group, were taken from culture vessels by sterile forceps and weighed in aseptic conditions. We used 0.3 g plant material from 3 biological samples for each groups. RNA isolation was performed according to the method described by Spiegel et al. (1996). The concentrations and purity of the isolated RNAs were determined by measuring OD260 & OD280 using a NanoDrop spectrophotometer (ThermoFisher Scientific), and the integrity of the RNAs was demonstrated by agarose gel electrophoresis. At the end, all samples were stored at -80°C until they were sent for RNA-Seq.

2.9.2. Sequencing library preparation

The TruSeq® Stranded Total RNA Sample Preparation Kit (Illumina) was used to prepare a library from total RNAs isolated from inoculated with active (A), inactive (I) PGB_invit and non inoculated control group (C) plants with 3 biological replicates. Paired-end sequencing with read lengths of 150 nt was carried

out on a HiSeq 4000 instrument (Illumina Inc., San Diego, California, USA).

2.9.3. Transcriptome sequence assembly and annotation

The raw data (at least 20 million read pairs for each sample) was processed as follows: first, quality control of the Illumina reads were carried out using FastQC (Andrews, 2010). Adapter regions and sequences with low quality reading were removed from the transcriptome sequences by using Lucy (Chou and Holmes, 2001), Pyrocleaner (Mariette et al., 2011), Figaro (White et al., 2008) and CLC Genomics Workbench (Liu et al., 2020) programs for a more accurate assembly. Overlapping paired-end reads were merged using the Flash program (Magoč and Salzberg, 2011) before assembling reads that were free of adapter and highly repeated sequences. This process facilitated the mapping of differently expressed genes to the reference genome. For reference-guided assembly, apple (*Malus x domestica* Borkh.) was selected the taxonomically closest species to Fraser's Photinia within the Rosaceae family for which a high-quality annotated sequence assembly was available. Therefore, the *M. domestica* cv. 'Golden Delicious' genome GDDH13 v 1.1 (Daccord et al., 2017) pseudomolecules and gene model annotations were downloaded from <https://iris.angers.inra.fr/gddh13>. Alignment to the reference and transcript quantification was also carried out using the HiSat2-StringTie-Ballgown pipeline (Pertea et al., 2016) with additional file processing using samtools 1.8. In order to account for differences in sequence between *P. x fraseri* genes and their orthologs in *M. domestica*, HiSat2 was run with the option `-score-min L,0,0.5`, permitting read alignments of >90% sequence identity. Only putative transcripts with at least 5x read coverage were included in subsequent analyses.

2.9.4. Gene Identification and Ontology

When assembled transcripts mapped to annotated genes in the *M. domestica* genome, the functional annotations from apple were transferred. All transcript contigs that did not map to annotated genes were used as queries for BLASTX in the UniProtKB/SwissProt database and NCBI non-redundant (nr) protein database. Contigs with an "E-value" of <1e-5 and showing more than 80% homology to known plant proteins, were identified as protein coding gene sequences. These sequences were scanned against the sequences found in the Blast2GO, Ontologizer 2.0 and UniProtKB databases to predict possible functions.

2.9.5. Determination of differentially expressed transcripts

The RNA-Seq and expression analysis modules included in the CLC Genomics Workbench program were used to perform the differential expression analysis, with additional analyses in Ballgown. High quality reads from each sample were mapped to the transcriptome assembly created above using CLC Genomics Workbench. For this alignment at least 95% read coverage was required with no more than two mismatches are allowed. The total number of mapped reads for each transcript was determined and normalized between samples using the FPKM (fragment per kilobase per million mapped reads) method. Transcripts with very low expression (average FPKM<2 across all samples) were eliminated. Statistical differences between the control and treatment groups were calculated using a false discovery rate (FDR) adjusted p-value<0.05. Statistically significant transcripts with an absolute fold change>2 between at least 2 of the test groups were determined as differentially expressed isoforms and genes, which were then are grouped into genes with increased or decreased expression after PGB_invit treatments. All data is shown as log2(FPKM+1) for each sample to check for sample-specific deviations.

2.9.6. Verification of RNA-Seq data with Real Time Quantitative RT-PCR (qRT-PCR)

The sequences of RNA-Seq were examined and candidate genes for qRT-PCR were determined to verify the differences in gene expression seen between trials. Primers designed for the selected genes are presented in Sup. Table 1. Confirmation of differently expressed genes identified by Illumina RNA-Seq with qRT-PCR method was performed using cDNAs (complementary DNA) synthesized by Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) from the same RNA extracts. Due to the highest expression levels, 15 genes were selected and these expressions were then compared with RNA-Seq results. qRT-PCR was performed by using StepOnePlus™ Real-Time PCR System (Applied Biosystems). As reference genes for qRT-PCR, 3 gene whose expression did not differ between trials as a result of RNA-Seq were chosen as candidates.

For qRT-PCR analysis, 0.9 µl (5 µM) per forward and reverse primer, 1 µl cDNA (500 ng), 7 µl 2x Maxima SYBR Green qPCR Master Mix (Thermo Scientific) and 5.2 µl sterile nuclease-free water were added (15 µl total volume) per each well into 96 well plate for both reference genes and for the genes to be analyzed. For each gene, 1 µl sterile nuclease-free water were added for negative control. The qRT-PCR conditions were as follows: initial denaturation at 95°C for 5 minutes and after that 40 cycles at 95°C for 15 seconds, 59°C for 30 seconds and 72°C for 30 seconds for each cycle. The melting curve was adjusted as 95°C for 15 seconds, 60°C for 1 minute and 95°C for 15 seconds. The fold change $2^{-\Delta\Delta Ct}$ calculated according to the C_t (cycle threshold) values of each gene were compared with three internal control genes. The data were evaluated with the statistical program.

2.10. Statistical Analyses

Analysis of all study results were done by using SPSS 12.0 program. The data were subjected to ANOVA to determine the differences between trials. The differences between the average data were subjected to LSD (least significant difference) multiple comparison test at level of $P \leq 0.05$. Proportional data were statistically evaluated by Chi-square test (χ^2). Heat-map graph of differently expressed genes among control and PGB_invit (active and inactive) inoculated groups was drawn using Microsoft Excel and GraphPad Prism 8 statistical programme based on the mean values of three replicates.

3. Results

3.1. *In vitro* proliferation of Fraser's Photinia

The results of *in vitro* proliferation of Fraser's Photinia microshoots which obtained after 30 days incubation on QL medium were presented in Table 1. Although, 38% of all sterilization trials were contaminated, 75-80% proliferation was obtained from sterile shoot tips. Additionally, multiple shoot formation was also observed. The lengths of microshoots were more than 3.5 mm. The proliferated shoots were subcultured in every 4 weeks for at least 3 months under *in vitro* conditions and then, they were used for bacterial inoculation experiments.

Table 1

Proliferation results obtained after 30 days cultivation of Fraser's Photinia microshoots transferred to *in vitro* conditions^a

	Contamination ^b (%)	Proliferation ^b (%)	Shoot Length ^c (M±SE) ^d (mm)	Shoot/explant ^c (M±SE) ^d	SFC ^e
1st Sterilization	38,80 ^a	80 ^a	3,93±0,19 ^a	1,58±0,12 ^a	1.26
2nd Sterilization	30,00 ^a	75 ^a	3,65±0,20 ^a	1,45±0,20 ^a	1.08
^a The data were collected 4 weeks after culture initiation. Each trial was done using at least 50 explants.					
^b The same letters in each column show no statistical difference ($P \geq 0.05$) compared to the Post-Hoc Multiple comparison test. The differences were evaluated vertically.					
^c The same letters in each column show no statistical difference ($P \geq 0.05$) compared to t-test. The differences were evaluated vertically.					
^d Mean ± standard error					
^e Shoot forming capacity (SFC)= Percentage of proliferating explants X The average number of shoots proliferated per explant/100 [37].					

3.2. Inoculation of Fraser's Photinia microshoots with PGB_invit and the determination of optimum inoculation concentration

At the end of the incubation for 30 days, it was seen that PSS did not have a negative effect on the proliferation of explants (Table 2). The 30 days incubation period of bacterial inoculation with both inactivated and active PGB_invit did not have a negative effect on the proliferation percentage of the explants, values of average shoot lengths, and the shoot/explant ratio as remarkably increased values were obtained with different incubation bacterium concentration. Elevation of shoot forming capacity [SFC=proliferation percentage. average shoot/explant ratio/100 (Lambardi et al., 1993)] was mostly achieved with inoculation of 10^2 and 10^4 cfu/ml of bacterial concentrations (1.80 and 1.82, respectively).

Table 2

The effect of PGB_invit on proliferation of Fraser's Photinia microshoots after 30, 45 and 60 days inoculation^a

The effect of PGB ₁ on proliferation of Fraser's Fuchsia microshoots after 30, 45 and 60 days inoculation											
	30 Days				45 Days				60 Days		
	Proliferation ^b (%)	Shoot/ explant ^c (M±SE) ^d	SFC ^e	Shoot Length ^c (M±SE) ^d (mm)	Proliferation ^b (%)	Shoot/ explant ^c (M±SE) ^d	SFC ^e	Shoot Length ^c (M±SE) ^d (mm)	Proliferation ^b (%)	Shoot/ explant ^c (M±SE) ^d	S
Control	75.00 ^b	1.40±0.13 ^b	1,05	3.00±0.29 ^{ab}	80.00 ^a	2.81±0.31 ^a	2,24	3.48±0.23 ^a	90.00 ^b	3.18±0.26 ^{ab}	2,
PSS	75.00 ^b	1.45±0.15 ^b	1,08	3.00±0.30 ^{ab}	55.00 ^c	3.36±0.52 ^a	1,84	3.43±0.19 ^a	66.66 ^e	2.80±0.24 ^b	1,
10 ² inactive	75.00 ^b	2.40±0.29 ^a	1,80	2.75±0.14 ^b	80.00 ^a	2.37±0.30 ^b	1,89	3.39±0.17 ^a	93.33 ^a	3.03±0.29 ^{ab}	2,
10 ⁴ inactive	80.00 ^a	2.43±0.81 ^a	1,94	3.24±0.16 ^a	75.00 ^{ab}	3.46±0.35 ^a	2,59	3.20±0.13 ^{ab}	90.00 ^b	3.11±0.28 ^{ab}	2,
10 ⁶ inactive	80.00 ^a	2.12±0.23 ^a	1,69	3.02±0.16 ^{ab}	75.00 ^{ab}	2.20±0.26 ^b	1,65	2.96±0.19 ^b	93.33 ^a	3.28±0.22 ^{ab}	3,
10 ² active	80.00 ^a	2.12±0.30 ^a	1,69	2.97±0.13 ^{ab}	80.00 ^a	2.94±0.30 ^a	2,35	2.97±0.10 ^b	76.66 ^d	2.73±0.19 ^b	2,
10 ⁴ active	75.00 ^b	2.20±0.20 ^a	1,65	3.18±0.21 ^{ab}	75.00 ^{ab}	2.93±0.45 ^a	2,19	3.59±0.17 ^a	80.00 ^c	3.60±0.30 ^a	2,
10 ⁶ active	60.00 ^c	2.08±0.33 ^a	1,24	3.08±0.22 ^{ab}	70.00 ^b	2.78±0.47 ^{ab}	1,94	3.20±0.16 ^{ab}	93.33 ^a	3.18±0.21 ^{ab}	2,
^a Each trial was done using at least 30 explants											
^b The different letters in each column show statistically significant differences (P≤0.05) compared to the Post-Hoc Multiple comparision test. The difference: vertically.											
^c The different letters in each column show statistically significant differences (P≤0.05) compared to LSD test. The differences were evaluated vertically.											
^d Mean ± standard error											
^e Shoot forming capacity (SFC)= Percentage of proliferating explants X The average number of shoots proliferated per explant/100 [37].											

The same positive effect was observed for shoot/explant ratio with bacterial inoculation period for 45 days (Table 2) and 60 days (Table 2). Although the highest shoot/explant ratio was found for the explants inoculated with active bacteria at a concentration of 10^4 for 60 days, the highest SFC ratio was found at 10^6 (2.96) among the active bacteria concentrations tested. The longest average shoot length (3.68 mm) was also measured at this concentration. For this reason, 10^6 was chosen as the optimum PGB_invit concentration that stimulates plant proliferation for bacterial trials that should be done with the active form. Same incubation concentration was also optimum for induction of proliferation of Fraser's Photinia microshoots after 60 days in inactive form (Table 2).

3.3. Determination the effect of PGB_invit on photosynthetic pigments

According to the results after 30 days incubation, there was no statistical difference in the amount of chlorophyll a, chlorophyll b and carotenoid of the microshoots inoculated with PGB_invit compared to the control group (Table 3).

Table 3
The effect of 30 days inoculation with 10^6 cfu/ml active PGB_invit on the concentration of photosynthetic pigments^a

	Chlorophyll a ^b (M±SE)	Chlorophyll b ^b (M±SE)	Carotenoid ^b (M±SE)
Control	0,11±0,02 ^a	0,11±0,03 ^a	0,03±0,004 ^a
PGB_invit	0,15±0,02 ^a	0,16±0,03 ^a	0,04±0,003 ^a
^a Each trial was done using at least 30 explants			
^b The different letters in each column show statistically significant differences ($P \leq 0.05$) compared to the t- test. The differences were evaluated vertically.			
^c Mean ± standard error.			

3.4. Determination of antioxidant enzyme activity

Although no difference was obtained in catalase activity of the active PGB_invit inoculated explants on 1st and 7th days of post-inoculation, it was statistically higher in 3rd day of post-inoculation in comparison to the control plants (Figure 1). In contrast, APX activity of microshoots of Fraser's Photinia inoculated with 10^6 cfu/ml active PGB_invit was decreased in only the first day compared to the control plants and it found statistically significant. In the other incubation days (3rd and 7th), there was no difference between the plant groups (Figure 1).

3.5. Determination of Proline Amount

On the first and third day of post-inoculation, proline amount was statistically higher in active PGB_invit inoculated samples than the control group. The plants inoculated with PGB_invit have no significant changes in amount of proline on the the 7th day while only 3rd and 7th days in the control group has been a slight increase in the control group (Table 4).

Table 4
Proline, MDA and H_2O_2 level differences of PGB_invit inoculated and control groups in the 1st, 3rd and 7th day of inoculation^a

	1st Day (M±SE) ^c		3rd Day (M±SE) ^c		7th Day (M±SE) ^c	
	Control	PGB_invit	Control	PGB_invit	Control	PGB_invit
Proline	103±14,0 ^a	140,5±17,5 ^b	130±8,0 ^a	150±2,0 ^b	142±8,0 ^a	152±2,0 ^a
MDA	116,6±16,5 ^a	147±9,5 ^a	133,1±15,7 ^a	155,8±14,4 ^a	202,3±37,0 ^a	176,5±25,0 ^a
H_2O_2	122,2±8,4 ^a	105,7±33,6 ^a	199,4±1,5 ^b	172,8±1,0 ^a	172±13,1 ^a	186,2±16,3 ^a
^a Each trial was done using at least 0.2 grams of explants						
^b The different letters in each row show statistically significant differences ($P \leq 0.05$) compared to the t- test. The differences were evaluated horizontally.						
^c Mean ± standard error						

3.6. Determination of Malondialdehyde (MDA) ve Hydrogen Peroxide (H_2O_2) Quantities

There was no statistically significant difference in the amount of MDA between the control and PGB_invit inoculated plants on days 1, 3 and 7 (Table 4). However, the amount of H_2O_2 increased on the 3rd day in control plants while it decreased in plants inoculated with PGB_invit (Table 4). There was no significant difference between the plant groups on the other days (1st and 7th).

3.7. Determination the effect of PGB_invit on rooting and *in vivo* acclimazitation of Fraser's Photinia

In IBA-free mediums, no rooting was observed in microshoots proliferated from bacterium inoculated plants and non-inoculated control plants. With the addition of 0.5 mg/l IBA to the medium, both control and bacteria-inoculated microshoots were rooted (55% and 60% respectively), (Table 5). Similar to the percentage of rooting, no statistical difference was recorded in the number of roots per microshoots and the average root length for both groups planted on 0.5 mg/l IBA containing medium (Figure 2). In addition, the percentages of survival after *in vivo* acclimatization of PGB_invit inoculated and control group plants was similar (80% and 75%, respectively) at the end of 30 days in greenhouse conditions.

Table 5
The effect of PGB_invit on rooting of Fraser's Photinia^a

Parameters	Rooting ^b (%)	Root/microshoot ^c (M±SE) ^d	Root length ^c (M±SE) ^d (mm)
IBA (0 mg/l)			
Control	0 ^b	0 ^b	0 ^b
10 ⁶ active PGB_invit	0 ^b	0 ^b	0 ^b
IBA (0.5 mg/l)			
Control	55.0 ^a	1.7±0.2 ^a	1.9±0.2 ^a
10 ⁶ active PGB_invit	60.0 ^a	1.5±0.3 ^a	2.0±0.4 ^a
^a The data were collected 4 weeks after culture initiation. Each trial was done using at least 30 microshoots..			
^b The different letters in each column show statistically significant differences (P≤0.05) compared to the Post-Hoc Multiple comparison test. The differences were evaluated vertically.			
^c The different letters in each column show statistically significant differences (P≤0.05) compared to LSD test. The differences were evaluated vertically.			
^d Mean ± standard error			

3.8. Monitoring localization of GFP-labelled bacterium in plant cells

After conjugation, GFP positive candidate colonies in Petri dishes were selected using the imaging system (Azure Biosystems, C600) and then positive transconjugants were also confirmed with the presence of the *gfp* gene by PCR amplification. In addition, PCR amplification was also carried out with *aux* primers to verify the presence of endophytic bacteria-specific auxin gene (*aux*) in the same colonies (Figure 3A).

Epiphytic and endophytic bacterial colonization was observed in the cross sections of Fraser's Photinia microshoots and it was visualized that the bacterium is endophytic (Figure 3B, 3C).

3.9. Determination of plant-PGB_invit interaction with molecular analyzes

Total RNA was isolated from the emerging microshoots of Fraser's Photinia explants that were inoculated with 10⁶ cfu/ml active or inactive PGB_invit, along with non-inoculated controls, after 60 days cultivation under *in vitro* conditions. RNA-seq analysis was carried out, and the total amount of raw data and the results of quality filtering are presented in Sup. Table 2. Unpaired reads, and paired reads with poor quality, were eliminated and not used in later stages. As can be seen in Sup. Table 2, for all samples >95% of reads were of good quality and sufficient to create the data of next analysis.

Sup. Table 3 and 4 shows the number of reads that were mapped to the reference genome/transcriptome for each of the samples in the experiment. As the reference genome (apple) was a different species, most reads did not map with very high percentage identity; as a result, only 35-41.9% of the total reads from Fraser's Photinia were mapped to the reference genome (Sup. Table 3) using CLC Genomics with default parameters. However, using HiSat2 with an empirically determined relaxed mapping quality score (corresponding to ≥ 90% sequence identity) 69.42-80.17% of read pairs were successfully mapped HiSat2 (Sup. Table 4). The similarities and differences in the distribution of gene expression between the two trials can be clearly seen when these figures are examined by evaluating these gene expression differences. According to gene expression levels (FPKM) from the CLC Genomics mapping results (Sup. Table 5), it was found that 35 of the 100 most abundant genes were differentially expressed among groups. 26 of these genes were upregulated and 5 of them were downregulated in active and inactive PGB_invit inoculated groups when compared to the control. From these 35 genes, 4 of them have expression levels which are varies (up or downregulated) among active and inactive PGB_invit inoculated groups when they compared to the control group. From all these differently expressed genes, XP_028953023, XP_008344383, XP_028943716, XP_008351175 and XP_028948163 genes were chosen to confirm expression differences by qRT-PCR.

Following the mapping according to HiSat2 and statistical analyses performed with Ballgown software, among the groups the 10 genes with the highest expression differences with >2-fold change in FPKM levels (Sup. Table 6) were selected to confirm gene expression by qRT-PCR. (Expression level changes of these genes are shown in Sup. Table 7).

BLAST results of these selected a total of 15 genes are also presented in Sup. Table 8. In addition, heat map of gene expression level changes of these genes presented in Figure 4. According to this results, one variant of the gene which is numbered with XP_008352422.1 (predicted "MID1-Complementing Activity 1") was upregulated and the other variant is downregulated in active PGB_invit inoculated group. 3 variants of XP_009355189.1 (Predicted "Two pore potassium

channel-1 like”) are upregulated and 2 variants are downregulated in inactive PGB_invit inoculated group. For XP_008385478.1 (Aspartic proteinase-like protein 2), one variant is upregulated and the other variant is downregulated in inactive group. One variant of XP_009359787.1 (uncharacterized ABC transporter-like) is upregulated both active and inactive PGB_invit inoculated groups and the other variant is have nearly the same expression levels in all groups. The gene numbered with XP_008375951.1 (Predicted “apoptosis inducing factor homolog B-like”) is upregulated both active and inactive PGB_invit inoculated groups but its expression level is higher in inactive group. KAB2620082.1 (RNA-dependent RNA polymerase I) have 6 variants and 3 of them is upregulated, 3 of them is downregulated in active and inactive PGB_invit inoculated groups compared to the control. 2 variants of XP_028948963.1 (Predicted “E3 ubiquitin-protein ligase RHA1B-like”) is upregulated and 1 variant is downregulated in active and inactive PGB_invit inoculated groups. PWZ54828.1 (Ubiquitin conjugating enzyme E2) have 2 variants and intrestingly one of them seen upregulated in all groups but expression levels are I, A, C, respectively, from highest to lowest. XP_028953023 (Methionine aminopeptidase 2B-like) is upregulated in active and inactive PGB_invit inoculated groups but its expression level is higher in the inactive group than active group. XP_008344383.2 (plasmodesmata-located protein 8) is upregulated in active PGB_invit inoculated group. XP_028943716 (UDP-glycosyltransferase 92A1-like isoform X2) seen upregulated in all groups but their expression levels are A, I, C, respectively, from highest to lowest. XP_008351175.2 (probable protein phosphatase 2C 34) is upregulated in active and inactive PGB_invit inoculated groups where it is downregulated in control group. And finally gene XP_028948163.1 (4-coumarate–CoA ligase-like 9) is upregulated in active and inactive PGB_invit inoculated groups but it has a higher expression level in inactive group than active group.

3.9.1. Verification of RNA-seq data by qRT-PCR

Of the selected 15 genes, gene bank ID XP_008352422.1 (predicted “MID1-complementing activity 1”), PWZ54828.1 (Ubiquitin conjugating enzyme E2), XP_028948963.1 (predicted “E3 ubiquitin-protein ligase RHA1B-like”), XP_008339965.2 (uncharacterized protein), XP_028948163.1 (4-coumarate-CoA ligase-like 9) XP_028953023 (methionine aminopeptidase 2B-like) expression levels were confirmed by qRT-PCR (Figure 5). The expression levels of XP_008352422.1, PWZ54828.1, XP_028948963.1, XP_008339965.2 genes decreased in plant samples inoculated with active and inactive bacteria compared to the control in accordance with RNA-seq results. Expressions levels of XP_028953023 and XP_028948163.1 genes increased in comparison to control in samples inoculated with active and inactive bacteria. However, XP_008375951.1, XP_009359787.1, XP_009338507.1, XP_008385478.1, XP_009355189.1, KAB2620082.1, XP_008351175.2, XP_008344383.2 and XP_028943716, which had expression differences in the samples inoculated with active bacteria compared to the control could not be verified with qRT-PCR because statistically no difference could be detected between trials (Sup. Figure 3).

4. Discussion

PGB_invit is reported previously to have nitrogen fixing ability and also GA3 and IAA producing capability (Gül Şeker et al., 2017). Hence, in our previous study (Batool et al., 2019), the inoculation of the *Arabidopsis thaliana* with the same PGB_invit bacteria, also showed that the PGB_invit culture has a positive effect on the proliferation of microshoots and multiple shoot formation. In accordance, these beneficial influences of PGB_invit on Fraser’s Photinia shoot proliferation were also evidenced in our study. Among the different concentrations of PGB_invit inoculated into Fraser’s Photinia shoot tips for various *in vitro* cultivation periods, 10⁶ cfu/ml found to be the most efficient one in active and inactive form. Moreover, the positive effects were observed especially in long incubation period (60 days) probably due to the slow generation time of PGB_invit. Besides, PGB_invit inoculation did not have any detrimental influence on rooting and *in vivo* acclimatization of Fraser’s Photinia microshoots. Moreover, our hypothesis, that PGB_invit can be an endophytic bacterium (Şah et al., 2019) is proven with the results presented in this study. Confocal microscopy photographs of cross sections of Fraser’s Photinia with the colonization of epiphytic and endophytic GFP-labeled PGB_invit shows clearly this in Figures 3B and 3C. The aim of this study was to elucidate the molecular mechanisms of Fraser’s Photinia and PGB_invit relationship.

Plants that encountered with biotic stress possess oxidative stress that occurs when there is a serious imbalance of production of reactive oxygen species (ROS) and antioxidant defense system (Anjum et al., 2012). The presence of high concentration of ROS in cells causes oxidative damage to photosynthetic functions and vital bio-molecules, and disrupt cellular metabolism. Therefore, photosynthetic pigments and MDA levels were determined in this study to reveal out if PGB_invit incubation leads to a serious oxidative stress in Fraser’s photinia *in vitro* cultures. Our results confirmed that there is no either any negative influence of this bacterium on photosynthetic pigments amount or distrupction of cellular membranes due to ROS formation.

Although no change in MDA levels obtained in this study, we still try to understand how Fraser’s Photinia respond to possible oxidative stress generated by PGB_invit in antioxidant level as plants have mechanisms to reduce ROS caused by biotic stress with antioxidant enzymes such as CAT, superoxide dismutase (SOD), GR, APX, and non-enzymatic antioxidants like ascorbic acid, proline and glutathione (Jankú et al., 2019). A decrease in H₂O₂ amount due to the increase in the activity of catalase enzyme on the 3rd day after inoculation of Fraser’s Photinia shoot tips was determined with 10⁶ cfu/ml active PGB_invit under *in vitro* conditions. All enzyme activity results showed that PGB_invit did not induce Fraser’s Photinia’s antioxidant mechanisms except catalase. Moreover, proline was also increased at the 1st day of inoculation, probably due to initial interaction. Overall biochemical results demonstrated that this bacterium does not cause major oxidative stress on its host and hence does not evoked alot antioxidant defence system.

As a result of our molecular analyses, we identified 45 genes in total that were differently expressed in active and inactive PGB_invit inoculated groups when compared to control group. From these 45 genes, 26 of them were upregulated, while 17 were downregulated in active and inactive PGB_invit inoculated groups. In addition, 6 of them had expression levels which varied (up or downregulated) between active and inactive PGB_invit inoculated groups. And finally, three of these 45 genes had expression level changes which varied according to different isoforms of the same gene transcript.

Among all the differently expressed genes, 15 of them were selected for qRT-PCR analysis and six of them were verified (Figure 5). Three of these genes (XP_028948963.1, XP_008339965.2, PWZ54828.1) expression were found to vary according to the isoform. This refers to the different use of transcript isoforms of the same gene depending on the conditions. However, since there is no database for the Fraser’s Photinia yet, studies based on Arabidopsis, maize and rice plants in the literature were used to evaluate the function of the last three genes (Sup. Table 7), whose gene expression varies according to isoform. In

the literature, RHA1B-like E3 ubiquitin-protein ligase gene is an enzyme investigated in Arabidopsis and rice and it has been reported that gene expression increases especially in biotic and abiotic stress conditions (Mazzucotelli et al., 2006). E3 ligases play an important role in post-translational modification as they define the substrate specificity of the ubiquitination complex (Diaz-Granados et al., 2020). Naturally, an increase in the expression of genes encoding this enzyme (XP_028948963.1) is expected in plant samples inoculated with active PGB_invit (Sup. Figure 2). Identified with the PWZ54828.1 genebank ID number Ubiquitin conjugating enzyme E2 (*Zea mays*) is involved in the protein ubiquitination mechanism; operates in tolerance to abiotic and biotic stress (Kim et al., 2005). According to studies in wild rice, the expression of the *OgUBC1* gene encoding E2 was increased when it was treated with injury and microbe-associated molecular motifs (MAMPs) and salicylic acid or exposed to UV-B radiation (Liu et al., 2020). Ectopic expression of the *OgUBC1* gene in Arabidopsis triggered the accumulation of anthocyanins in the leaves of the genes associated with defense and stress. In addition, these plants expressing the gene in question are resistant to UV-B related cell damage and *Botrytis cinerea* infection. In this study, while the gene in question has to increase its expression due to the biotic stress factors applied under *in vitro* conditions, its expression has decreased in the samples inoculated with both active and inactive PGB_invit compared to the control samples. This situation shows that the inoculation with PGB_invit does not cause stress on the Fraser's Photinia once again.

In addition, according to BLAST results, we performed qRT-PCR analysis to verify gene expression level changes and in the result of this RNA-Seq and qRT-PCR had been performed on Fraser's Photinia for the first time in our study.

According to the qRT-PCR results, the genes named with XP_008352422.1, XP_028948163.1 and XP_028953023 were found out that they are coding mid1-complementary activity (MCA) protein, 4-coumarate-CoA ligase and methionine amino protease (MetAP2), respectively and their expression level changes were verified with qRT-PCR. The mid1-complementary activity (MCA) protein family are found in plasma membrane and shown to function as Ca^{2+} -permeable mechanosensitive channels. They participate in Ca^{2+} uptake at the roots. MCA channels appear to have common functions in plants, based on studies on *Oryza sativa* OsMCA1, *Nicotiana tabacum* NtMCA1, NtMCA2, *Zea mays* CNR13 (Nishii et al., 2021). Experiments with various cell types have shown that increasing the expression of the MCA gene increases Ca^{+2} uptake under normal and membrane-disrupting conditions (Nakano et al., 2011). Furthermore, the reduction of MCA in Fraser's Photinia plants inoculated with active PGB_invit shows that the biotic agent does not increase Ca^{+2} uptake into the cell as it does not cause stress in the plant.

Expression of 4-coumarate-CoA ligase is optimized at developmental stages and in response to biotic and abiotic stresses (Lavhale et al., 2018). The 4-coumarat-CoA ligase-like 9 gene plays a role in the conversion of α -linolenic acid to jasmonic acid (JA). Therefore, this protein is seen as an enzyme involved in the activation of JA biosynthesis. It is known that jasmonic acid is one of the two main plant growth regulators that play a role in regulating the plant defense system against biotic and abiotic stresses while the complex interactions of jasmonic acid with salicylic acid, other plant growth regulators such as ABA and cytokinins also play a role (Robert-Seilanianantz et al., 2011; Erb et al., 2012). In this respect, the increase of gene expression compared to the control of the gene of interest in plant samples inoculated with active and inactive PGB_invit shows that this bacterium activates JA biosynthesis in the plant.

Methionine amino protease (MetAP2) whose gene expression is increased in plants inoculated with active and inactive PGB_invit compared to control, is the most important component of the N-terminal methionine extraction (NME) pathway that removes the first methionine of proteins and catalyzes the basic post-translational modification. N-terminal methionine extraction is the main universal process, protected from eubacteria to higher eukaryotes. NME is regulated by stress (Gigliione et al., 2004) and it is well known that it is necessary for proper plant growth in Arabidopsis (Ross et al., 2005). Although there is not much publication about the effects of MAP in response to stress in plants, it has been reported that a new MAP protein (HvMAP) in barley has increased gene expression in samples treated with low temperature (Jeong et al., 2011). In addition, transgenic Arabidopsis lines expressing this gene in large amounts have been observed to have a relatively strong freezing tolerance compared to wild types. These results suggest that, HvMAP functions in freezing tolerance by facilitating protein maturation in the conditioning process. In this study, an increase in the expression of this gene in the plants inoculated with active and inactive PGB_invit may be associated with biotic agent inducing plant growth by causing a large amount of protein to mature.

5. Conclusion

In summary, physiological, biochemical and molecular effects of the PGB_invit are evaluated together on the Fraser's Photinia plant under *in vitro* conditions and optimum PGB_invit concentration that increases *in vitro* proliferation of Fraser's Photinia is determined as 10^6 cfu/ml. PGB_invit inoculation relatively increased the amount of chlorophyll and did not cause a remarkable oxidative stress. It also has been confirmed that the localization of the PGB_invit in question is endophytic. According to RNA-seq results of Fraser's Photinia plants inoculated with active bacteria, the induction or reduction of expression of several genes [gene bank ID XP_008352422.1 (predicted "MID1-complement activity 1"), PWZ54828.1 (ubiquitin conjugated enzyme E2), XP_028948963.1 (predicted "E3 ubiquitin-protein ligase" RHA1B-like"), XP_008339965.2 (uncharacterized protein), XP_028948163 (4-coumarat-CoA ligase-like 9), XP_028953023 (methionine aminopeptidase 2B-like) were verified by qRT-PCR. It has been identified three internal control genes to be used for plant-PGB_invit interaction [XP_008338169.1 (ubc12-like NEDD-conjugating enzyme, VVA10186.1 (20 kDa predicted chaperone), XP_017186573.2 (50S ribosomal protein L28, chloroplastic like) for the first time in Fraser's Photinia.

Our results in this study together with our previous studies have been demonstrated that PGB_invit has a positive impact on plants growth and development with several mechanisms. Besides its shown that PGB_invit is not act as a stress factor, as it does not cause a severe oxidative stress and thus can be used as a biofertilizer.

Declarations

DECLARATION OF COMPETING INTEREST

The authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any author. Sponsors did not have any role in study design; the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

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Competing Interests

All Authors declare they have no financial interests.

Author Contributions

NT, MK, HE, conducted the research, YÖÇ, ÖA, MGŞ, CK planned all the experiments, SJL carried out bioinformatic analysis, All of the authors contributed to the writing of the MS.

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Figures

Figure 1

Antioxidant enzyme activities of control and active PGB_invit inoculated group. Catalase activity significantly increased in the 3rd day and ascorbat peroxidase activity is significantly decreased in the 1st day in active PGB_invit inoculated group.

Figure 2

The rooting of microshoots inoculated with 10^6 cfu/ml PGB_invit in 0.5 mg/l IBA supplemented medium.

Figure 3

A. 1% agarose gel image after PCR amplification of transconjugants using aux and gfp primers 1. aux gene ~750 bp, 2. negative control, 3. DNA ladder (1000 bp, Intron, 24074), 4. E. coli DH5α strain with pSEVA237-G plasmid containing gfp gene and 5. ~750 bp gfp gene result obtained from the amplification of PGB_invit broth culture samples using aux primers, 6. negative control. **B, C.** Colonization of epiphytic and endophytic GFP-labeled PGB_invit in the cross sections of Fraser's Photinia.

Figure 4

The heat map shows the differently expressed genes among control and PGB_invit (active and inactive) inoculated groups. The expression levels of genes are plotted as $\log_2(\text{FPKM}+1)$ for each sample to check for sample-specific deviations. The log conversion normalizes all data to a similar extent. In order to visualize the zero values in the graph, 1 is added to all log values. The up-regulated genes are indicated in red and down-regulated genes in green. The intensity of the color increases as the expression differences increase as shown in the bar at the right.

Figure 5

Expression results of genes whose gene expression was confirmed by qRT-PCR. XP_008338169.1, VVA10186.1 and XP_017186573.2 represent 3 different internal control gene, XP_008352422.1, PWZ54828.1, XP_028948963.1, XP_008339965.2, XP_028948163 and XP_028953023 represents the target genes. The expression levels of genes were converted to 2^{-ΔΔCt} format compared to the control and presented using fold change values. C: Non-inoculated 60 days old photinia microshoots as control, A: 10⁶ active PGB_invit inoculated 60 days old photinia microshoots, I: 10⁶ inactive PGB_invit inoculated 60 days old photinia microshoots. Bars represent the standard error, and the different letters represent significant differences for each group (P<0.05).

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