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Short communication

Vir gene inducers in *Dunaliella salina*; an insight in to the *Agrobacterium*-mediated genetic transformation of microalgae

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ABSTRACT

Phenolic compounds such as vanillin, vanillic acid, gallic acid, coumarin, caffeic acid and acetosyringone, extracted and identified from spent medium of *Dunaliella salina* has shown to facilitate the virulence (*vir*) gene induction in *Agrobacterium* strain A348 (harboring pSM358, a virE::LacZ fusion vector). Among these phenolic compounds, vanillin showed highest concentration of 38.12 ± 0.88 μg L^{-1} in spent medium. All these phenolic compounds were evaluated independently for induction of *vir* gene expression using β-galactosidase activity. Our results showed that except for gallic acid, there was significant *vir* gene induction activity and these phenolic compounds may be involved in facilitating efficient *Agrobacterium*-mediated transformation in *D. salina*.

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1. Introduction

Agrobacterium-mediated transformation has proven to be efficient system in constructing genetically modified organisms (GMOs) [1]. This method has several advantages over other direct transformation methods which include stability, inexpensive and simple procedure, integration of large DNA fragments with little rearrangements in the host genome and wide host range [2,3]. Recent studies involving genetic manipulation among microalgae through the *Agrobacterium*-mediated transformation has been demonstrated in different species such as *Chlamydomonas reinhardtii* [4,5], *Haematococcus pluvialis* [6], *Schizochytrium* [7], *Chlorella vulgaris* [8], *Nannochloropsis* [2], *Scenedesmus almeriensis* [9], and *Dunaliella* [10].

The transformation process involves complex machinery of *Agrobacterium* and host organism. The major genetic factors needed include the T-DNA portion and the virulence (*vir*) region of the Ti plasmid of *Agrobacterium*. The virulence genes (*vir* genes) in the *vir* region of Ti plasmid facilitate the transfer of T-DNA portion to the target organism [1]. Different low molecular weight compounds such as acetosyringone, cinnamic acid, vanillin etc. produced as a part of defence mechanism, in plants, are recognized by the *Agrobacterium* and used as a signal for *vir* gene induction for T-DNA transfer [11–14]. It is also reported that synergistic action of phenolic compounds and sugar molecules such as galactose, glucose, group of aldose sugars etc. helps the induction of *vir* gene of *Agrobacterium* in plants [15,16]. Acetosyringone (3,5-dimethoxyacetophenone, AS) is the major phenolic compound identified as the *vir* gene inducer [17]. However, from our previous studies, it was found that *Agrobacterium*-mediated genetic transformation in *Dunaliella* does not require the presence of acetosyringone [10]. The co-cultivation of *Dunaliella* in the absence of acetosyringone consistently produced reporter gene expression with transformation frequency similar to that with acetosyringone [10]. Similar observation of *Agrobacterium*-mediated genetic transformation was also reported in *C. reinhardtii* [4] and *H. pluvialis* [6]. *Chlamydomonas* was investigated previously for its ability to induce *Agrobacterium vir* gene, that is essential for T-DNA transfer [18]. Previous reports have not discussed the type of compounds produced by microalgae that are responsible for *vir* gene induction in *Agrobacterium*. In the present study, we focused on the compounds from microalga *Dunaliella salina*, that seems to be responsible for *vir* gene induction.

2. Materials and methods

2.1. Algal and bacterial culture maintenance

D. salina strain V-101 was obtained from Centre for Advanced Studies in Botany, University of Madras, Chennai, India. Liquid cultures of *D. salina* were maintained in modified AS100 (MAS 100) medium [10]. For co-cultivation, MAS 100 and TAP media [19] containing 0.1 M sodium chloride were used to sustain the growth of *D. salina*. The

Abbreviations: TAP, tris acetate phosphate medium; ASM, algal spent medium; ACP, algal cell pellet; BCP, bacterial cell pellet.

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cultures were maintained at light intensity of 18.75 ± 2.5 μmol m^{-2} s^{-1} for 16 h a day at 25 ± 1 °C and mixed manually once a day.

Agrobacterium tumefaciens strain EHA-101, transformed with the binary vector pCAMBIA 1304 [10] was used for the initial co-cultivation with *D. salina* to find out the effect of co-cultivation in the *vir* gene induction. Another *A. tumefaciens* strain A348, harboring pSM 358 (a *virE::lacZ* fusion vector), obtained from the University of Washington, was used for checking the *vir* gene induction activity. This strain of *A. tumefaciens* contains a *virE::lacZ* fusion vector, where the *lacZ* gene that encodes β-galactosidase is placed under the control of *vir* gene locus. So the induction of *vir* region was monitored by measuring the β-galactosidase activity [20]. *A. tumefaciens* cultures were maintained on Luria–Bertani (LB) medium containing kanamycin (100 mg L^{-1}) and rifampicin (50 mg L^{-1}).

2.2. Co-cultivation of *D. salina* and *A. tumefaciens*

D. salina culture was freshly inoculated in to the liquid medium from a stock culture on slants. The culture was grown for a period of 10 days, and the log phase culture was harvested and resuspended in liquid medium (TAP and MAS 100) for bringing the cell density of 1.5×10^4 cells mL^{-1}. *A. tumefaciens* harboring pCAMBIA 1304 was also freshly inoculated in LB medium and was incubated overnight at 200 rpm at 28 °C. The culture was transferred to 50 mL LB medium on the next day and incubated till optimum OD_{600} reaches to 0.5. The *A. tumefaciens* cells were harvested and resuspended in 2 mL liquid TAP and MAS 100 medium. *D. salina* culture (100 mL) was co-cultivated with freshly grown culture of *A. tumefaciens* (200 μL) in liquid TAP medium for 48 h. Controls were also maintained without the addition of *A. tumefaciens* in both media. After 48 h, the culture was centrifuged at 1000 rpm to separate the algal cells from the supernatant (referred as algal cell pellet — ACP). The supernatant that contained *A. tumefaciens* cells was again centrifuged at 5000 rpm to separate the *A. tumefaciens* cells (referred as bacterial cell pellet — BCP) and the spent medium (referred as algal spent medium—ASM).

2.3. Induction of *vir* gene in Agrobacterium

In the first experimental set, the samples used in the study were ASM and ACP from co-cultivated and controls followed by BCP from both co-cultivated cultures (in TAP and MAS 100 media). For ACP and BCP, cultures were pelleted, and cells were re-suspended in 5 mL fresh medium and lysed by sonication and the cell debris were removed by centrifugation at 12,000 rpm for 10 min at 25 °C. The supernatant was made up to the original volume and used as induction medium for *A. tumefaciens*. For the second experimental set, ASM at different time intervals (3, 5, 7 and 10 days) in both modified TAP and MAS 100 media were used for *vir* gene induction in *A. tumefaciens*. For the third set of *vir* gene induction analysis, total phenolic compounds were extracted from 3rd, 5th, 7th and 10th day old cultures and the extracts were dissolved in DMSO (Sigma Aldrich Bangalore) and added to the MAS 100. For the fourth set of experiment, standard phenolic compounds (acetosyringone, vanillin, vanillic acid, coumarin, gallic acid, caffeic acid) obtained from Sigma Aldrich Bangalore were added individually (100 μM) in MAS 100 and used as induction medium. *A. tumefaciens* culture harboring pSM 358 was grown overnight at 200 rpm at 28 °C in LB medium in an incubator shaker. The culture was centrifuged for 5 min at 8000 rpm at 24 °C. The pellet was re-suspended in 15 mL of different induction media as mentioned above. The *A. tumefaciens* strain A348 was inoculated at an initial OD of 0.5 at A_{600} and incubated at 25 °C with 120 rpm for 48 h. After 48 h, cultures were centrifuged at 10,000 rpm for 10 min at 24 °C and the cell pellets were used for β-galactosidase assay [21].

Table 1
β-galactosidase activity of spent media and cell extract of *D. salina* and *A. tumefaciens*.

Sample	β-galactosidase activity (miller units)*	
	TAP	MAS100
ACP	62.1 ± 5.7[b]	69.7 ± 4.6[b]
ASM	175.5 ± 5.98[a]	186.23 ± 10.4[a]
CoACP	53.6 ± 7.2[b]	75.4 ± 5.7[b]
CoASM	165.76 ± 9[a]	190.33 ± 3[a]
BCP	9.53 ± 2.6[c]	22.3 ± 3.2[c]
Media	9.5 ± 1.86[c]	12.3 ± 0.87[c]

*: Values are mean ± standard deviation, significance was tested by Duncan Multiple Range Test at $p < 0.05$, and values with same superscript were found not significantly different from each other. ACP—algal cell pellet, ASM—algal spent medium, BCP—bacterial cell pellet, Co—co-cultivated.

2.4. Extraction of phenolic compounds

Total phenolic compounds were extracted from ACP, BCP and ASM using ethyl acetate. 20 mL ethyl acetate was thoroughly mixed with ASM (100 mL) and the solvent containing phenolics was collected. Total phenolics from ACP and BCP were extracted by disrupting the cells in mortar and pestle using ethyl acetate as solvent. The disrupted cells were centrifuged at 10,000 rpm for 10 min at 24 °C and the solvent phase was separated. The extraction was repeated and the solvent phases were pooled and dried separately using a rotavapour (R205 V800, Buchi Labortechnik, Flawil, Switzerland). All the dried samples (ASM, ACP and BCP) were dissolved separately in known final volume of methanol.

2.5. Estimation of total phenolic compounds

Total phenolic content was estimated by the modified Folin–Ciocalteu method [22]. 200 μL of diluted sample was added to 1 mL of 1:10 diluted Folin–Ciocalteu reagent. After 5 min, 800 μL of saturated sodium carbonate (75 g L^{-1}) was added and incubated for 30 min at 37 °C. The absorbance at 750 nm was measured for determining total phenolics in spectrophotometer (UV-1800 240 V, Shimadzu, Kyoto, Japan). Gallic acid was used to prepare standard calibration curve.

2.6. HPLC analysis

The chromatographic analysis of phenolic compounds was performed in Shimadzu HPLC instrument (LC 10A, Shimadzu, Kyoto, Japan) equipped with a UV/Vis detector, using a reverse phase C-18 column (15 cm × 4.6 mm of 10 μm particle size; Waters, Manchester, UK) at a flow rate of 1 mL min^{-1}. The mobile phase consisted of solvent A, 0.5% acetic acid; solvent B methanol. The gradient program applied was: 0 min, 90% A; 26.15 min, 60% A; 30 min, 90% A [23]. Detection was carried out at 290 nm and identification and quantification were carried out by comparing with standards (Sigma Aldrich, Bangalore).

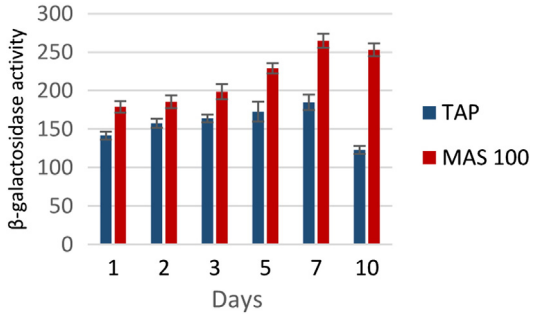

Fig. 1. *Vir* gene induction activity in spent medium of *D. salina* at different intervals of time (in TAP and MAS 100 media).

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