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MICROSCOPIC OBSERVATION OF A ROOT-ADHERING FACTOR Avin_16040 IN *Azotobacter vinelandii* AND *Escherichia coli* DURING ASSOCIATION WITH RICE ROOT

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ABSTRACT

Microbial adherence to plant root is the initial step in a beneficial plant-microbe interaction. Quantitative RT-PCR analysis deduced the *Avin_16040* gene showed upregulated expression when *Azotobacter vinelandii* was adhered to the rice root. By transforming the full-length *Avin_16040* gene into a heterologous host *Escherichia coli*, the recombinant clones displayed filamentous cell shapes in contrast to the rod-shape of wild type cells. Besides full-length gene insert, some *E. coli* clones were detected to contain truncated *Avin_16040* gene inserts but still shape-shifted to filamentous cells. Further analysis by DNA sequencing revealed the shape-shifting *E. coli* clones contained 3'-end truncated *Avin_16040* gene, while *E. coli* clones containing the 5'-end truncated *Avin_16040* gene remained rod-shaped. The cell surface topographies of *A. vinelandii* and *E. coli* cells in the presence and absence of *Avin_16040* gene and in association with rice root adherence were analysed using atomic force microscopy.

Keywords: Avin_16040, *Azotobacter vinelandii*, *Escherichia coli*, AFM

INTRODUCTION

Azotobacter vinelandii is a plant-growth-promoting bacterium commonly found in soil. It is well known as a dinitrogen fixer, as well as a plant growth hormone producer. Besides, *A. vinelandii* has a long research history of biosynthesizing the extracellular polysaccharide alginate, the intracellular polyester poly-β-hydroxybutyrate (PHB) and siderophores which are compounds that were reported to have multiple biotechnology and biomedical applications. These applications include alginate for control release of medical drugs (Yao *et al.*, 2009) and as food additives (thickener, stabilizer, gelling agent and emulsifier), polyhydroxybutyrate (PHB) for development of biodegradable and biocompatible thermoplastics (Diaz-Barrera & Soto, 2010), and siderophores as drug delivery (Möllmann *et al.*, 2009), antimicrobial (Upadhyay & Srivastava, 2008) and soil bioremediation agents (Braud *et al.*, 2009).

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Plant-growth-promoting rhizobacteria (PGPR) can improve the overall development of plants by increasing its root and shoot yields (Zakry *et al.*, 2010). As a PGPR, *A. vinelandii*'s N_2 fixation has been extensively studied since 1960s. Other PGPR characteristics most commonly researched into are its ability to produce plant growth hormone and amino acids. Torres-Rubio *et al.* (2000) reported that *A. vinelandii* isolated from a rice rhizosphere produced the highest concentration of indole-3-acetic acid (IAA) in comparison to the other isolates residing at the same rhizosphere, namely *A. chroococcum, Pseudomonas aeruginosa*, *P. putida* and *Serratia* sp. Rodelas *et al.* (1999) and Pozo *et al.* (2000) discussed the production of plant-growth-promoting amino acids by *A. vinelandii* and *A. chroococcum*. *A. vinelandii* ATCC 12837 was also reported to colonize the rhizospheres of cereal (Shimshick & Hebert, 1979), rice (Maudinas *et al.*, 1981; Torres-Rubio *et al.*, 2000), wheat (Naz *et al.*, 2012) and hot pepper (Husen, 2005). This non-endosymbiont root association has brought about supply of biologically fixed N_2 to the plant,

enhanced soluble P availability, antibiosis against pathogenic microorganisms, as well as supplies of plant-growth-promoting hormones, vitamins and amino acids to the plant (Okon & Itzigsohn, 1995; González López *et al.*, 1999; Revillas *et al.*, 2005). Concomitantly, root-colonizing *A. vinelandii* can incorporate readily the plant root exudates which may contain sugars and organic acids.

Recently, we reported that a surface layer protein Avin_16040 was involved in the adherence of *A. vinelandii* ATCC 12837 cells to rice root (Liew *et al.*, 2015). In the same study, we observed that the heterologous host *E. coli* showed increased adherence to rice root after it was transformed with *Avin_16040* gene. This paper aims to impart differing cell surface topographies of *A. vinelandii* and *E. coli* cells in the presence and absence of *Avin_16040* gene in relation to root adherence. For this purpose, atomic force microscopy (AFM) was used. Besides, DNA cloning of *Avin_16040* gene in *E. coli* will be discussed by emphasizing that the *E. coli* clones which contain 3'-end truncated *Avin_16040* gene also displayed "filamentous cell" effect as the *E. coli* clones which contained full-length *Avin_16040* gene.

MATERIALS AND METHODS

Bacterial strains, plasmid and culture conditions

Azotobacter vinelandii Lipman ATCC 12837 was obtained from the American Type Culture Collection (ATCC). Deletion mutant *A. vinelandii* Δ*Avin_16040* was described previously by Liew *et al.* (2015). Both bacterial strains were maintained and grown in Burk's medium containing 2% (w/v) sucrose. Liquid cultures were agitated continuously at 200 rpm, 25 ± 2°C, for up to 5 days. Agar medium was solidified with 2% Agar Bacteriological No.1 (Oxoid, UK). *A. vinelandii*-root interaction was performed in altered Murashige and Skoog (1967) medium as described before (Liew *et al.*, 2015). *E. coli* DH5α was cultivated in Nutrient Broth (NB) (Merck, USA) for overnight at 37 ± 2°C.

Sample preparation and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

O. sativa MR 219 seeds were surface sterilized and germinated as described by Liew *et al.* (2015). Axenic roots of *O. sativa* MR 219 were interacted with *A. vinelandii* ATCC 12837 wild type in three systems; (1) adventitious roots were aseptically excised from the rice seedlings with scalpel blade and cut into short fragments of approximately 1 cm before they were mixed separately with ATCC 12837 cells at 10^7 CFU/mL concentrations in the altered-MS medium, (2) adventitious roots were shredded and vortexed vigorously in the altered-MS medium, after which shredded roots were harvested and re-suspended in fresh medium containing ATCC 12837 cells as described for 1 cm root fragments, (3) root extract (supernatant) was interacted separately with ATCC 12837 cells. Root-microbe interaction was performed for 2 hrs at static condition. The positive control was constructed by mixing the ATCC 12837 cells with the roots of the rice seedlings. Root-adhered ATCC 12837 cells were lysed immediately in cell lysis solution and processed for RNA extraction. RNAs were also prepared from the free-floating cells (not adhered to roots) in the root-microbe interaction systems as well as non-root-interacted bacterial culture (negative control). To verify the expression of Avin_16040, root-adhered ATCC 12837 cells were analysed after 0 hr, 10 min, 20 min, 30 min, 1 hr, 2 hrs, 4 hrs, 8 hrs and 24 hrs of root-microbe interaction. RNA extraction, reverse transcription and qRT-PCR were performed according to Liew *et al.* (2015).

DNA manipulations and analyses

The DNA fragment encoding the *Avin_16040* gene was ligated to the plasmid vector pJET1.2/blunt (Fermentas, Lithuania) before the mixture was transformed into *E. coli* DH5α. Bacterial cells were smeared on glass slides and observed under Primo Star upright microscope (Zeiss, USA). DNA inserts were amplified by PCR. *E. coli* clones carrying different DNA insert sizes were selected for further analysis. DNA sequencing was performed commercially.

AFM

Atomic force microscopy (AFM) was conducted using JPKNanoWizard II system (JPK Instruments AG, Germany) to display bacterial cell structure and cell surface topography. Bacterial smears were used for the purpose. The bacterial smears were prepared according to Liew *et al.* (2015).

RESULTS AND DISCUSSION

The bacterial strain *A. vinelandii* ATCC 12837 was studied during its interaction with *O. sativa* MR 219 root and a hypothetical protein Avin_16040 was identified via a 2-dimensional gel electrophoresis – tandem mass spectrometry (2DE-MS/MS) method (Liew *et al.*, 2015). The protein was found to show an elevated expression level during close contact with *O. sativa* MR 219 roots. An Avin_16040 deletion mutant was then generated, which revealed some interesting aspects on the functions encoded by the protein coding gene.

In this paper, we present additional data on the hypothetical gene coding for Avin_16040 as well as on the characteristics of its deletion mutant *A. vinelandii* Δ*Avin_16040*, especially in relation to the root adherence function. Preliminarily, the *in vitro* interaction between *A. vinelandii* and *O. sativa* was conducted by subjecting the bacterial cells to roots of the *O. sativa* seedlings. To explore whether the root (physical effect) or root exudates (chemical effect) caused the elevated expression of Avin_16040, *A. vinelandii* was interacted with roots that were cut into short fragments of 1 cm in length, shredded roots, and root extracts. Root extract was obtained by separating the medium supernatant from the shredded root debris after vigorous vortexing. Relative to the cells at 0 hour, *Avin_16040* gene showed an upregulated expression in the bacterial cells which resided on *O. sativa* roots of the seedlings, as well as on the root fragments of 1 cm in length (Figure 1). No significant change in expression levels was detected in the interactions with root extract and shredded roots. The results indicated that the elevated expression of *Avin_16040* gene was caused by *A. vinelandii*'s adherence to root (physical effect). Further analysis at selected time points revealed the *Avin_16040* gene responded almost immediately to the root (fragment)-microbe interaction. Figure 2 shows that its expression started to escalate after only 10 min interaction with roots. By supplementing nitrogen (1.65 g L^{-1} ammonium nitrate) to the interaction medium, *Avin_16040* gene showed lower expression level in general. The ability to colonize plant root is an important characteristic of plant-beneficial

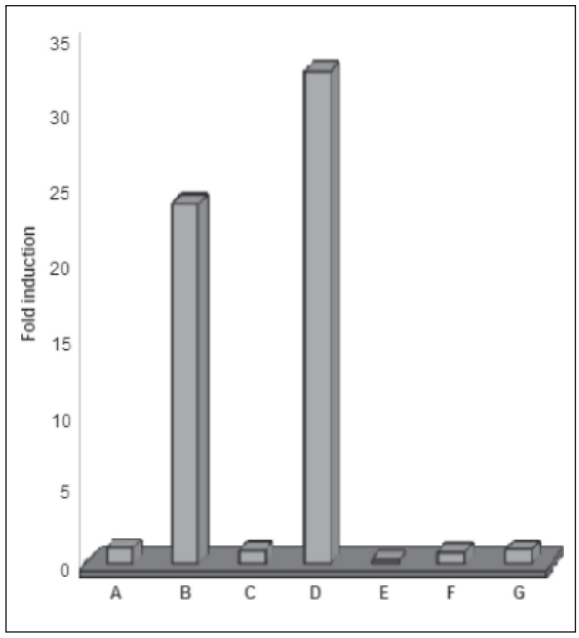


Fig. 1. Comparison of *Avin_16040* gene expression extracted from different *O. sativa* roots – *A. vinelandii* interaction conditions. Bacterial cells were interacted with roots for 2 hrs at static condition. The results showed *Avin_16040* gene was upregulated in *A. vinelandii* attached to *O. sativa* roots. A, *A. vinelandii* cells at 0 hr, B, root-attached bacterial cells after 2-hr interaction with *O. sativa* root attached to seedlings, C, free-floating bacterial cells after 2-hr interaction with *O. sativa* root attached to seedlings, D, root-attached bacterial cells after 2-hr interaction with cut *O. sativa* root fragment, E, free-floating bacterial cells after 2-hr interaction with cut *O. sativa* root strands, F, bacterial cells after 2-hr interaction with *O. sativa* root extract, G, bacterial cells after 2-hr interaction with *O. sativa* roots shredded with scalpel blade.

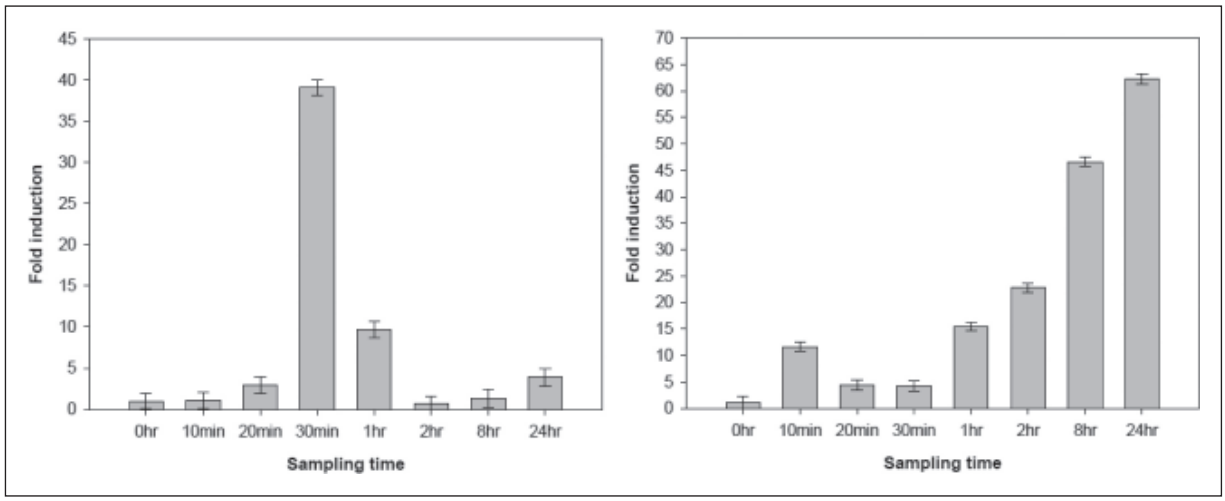


Fig. 2. Quantitative RT-PCR analysis of *A. vinelandii* ATCC 12837 during root adherence in altered Murashige and Skoog (1967) medium containing nitrogen (left) and devoid of nitrogen (right). The expression level of *Avin_16040* gene was normalized against the 16S rRNA gene (Liew *et al.*, 2015). In the medium containing nitrogen, *Avin_16040*'s expression fluctuated where it spiked upon 30 min of *A. vinelandii* – *O. sativa* root interaction before decreased, but detected slight increase after 24 hrs of interaction. In contrast, the expression of *Avin_16040* gene increased steadily after 30 min of interaction until the experiment was stopped at 24-hr.

bacteria. This close proximity between microbe and plant root implied that the microbe could directly affect the performance of plant via mutual exchange of signal molecules such as phytohormones and nutrients, as well as sugars and amino acids (Brechenmacher *et al.*, 2010). This association resembles a symbiotic relationship.

Using AFM, the cell shapes and structures of *A. vinelandii* during interaction with *O. sativa* roots were observed (Figure 3). In general, both Δ*Avin_16040* deletion mutant and ATCC 12837 strains showed the formation of "thread-like" networks during root adherence. However, the deletion mutant displayed "grainy" motif which is narrower in width than the "filamentous" motif of the ATCC 12837 wild type. In addition to the "grainy" motif, the root-adhered deletion mutant also displayed "patches" motif which were most possibly amassed from groups of cells as viewed under the compound microscope (Fig. 3E). The different motif formation directly reflected the decreased root adherence capacity of Δ*Avin_16040* which was reported previously by Liew *et al* (2015). AFM analysis of the non-root-interacted *A. vinelandii* cells revealed Δ*Avin_16040* cells were generally bigger than the ATCC 12837 cells. By performing electron microscopy analysis, Shimshick and Hebert (1979) reported that *A. vinelandii* ATCC 12837 colonizes plant root in both monolayer and multilayer formats until 10^9 cells per g root. Even though the majority of ATCC 12837 cells adhered to root individually, some adhered in patches of several hundred cells with considerable overlaps (Shimshick & Hebert, 1979).

The DNA fragment of *Avin_16040* gene was cloned in pJET1.2/blunt plasmid vector and transformed into *E. coli* DH5α. By performing PCR analyses, recombinant *E. coli* clones were found to contain several DNA insert sizes and were further analysed. DNA sequencing analysis showed that partial *Avin_16040* genes with 3'-end truncation and 5'-end truncation were cloned (Figure 4). The cause and mechanism that brought about the insert size variation was unknown. However, it provided additional information which was useful in the genetic mapping of S-layer proteins. Translation of a recombinant protein especially by a high copy number plasmid might impose a metabolic burden that decreases the growth rate of bacterial host and affects plasmid instability (Bentley *et al.*, 1990; Birnbaum & Bailey, 1991). In such an occurrence, truncation of the recombinant gene might be a successive effect. The recombinant clones carrying different *Avin_16040* gene fragments were selected for cell morphology analysis using a compound microscope. The *E. coli* clones (represented by clone 58) which were transformed with the full-length *Avin_16040* gene (1,368 bp) had cell shapes which were filamentous (Figure 5). Interestingly, the *E. coli* clones containing 3'-end truncated *Avin_16040* genes of 1,256 bp (clone 84) and 346 bp (clone 27) in length were also displaying filamentous cell morphology. In contrast, an *E. coli* clone (clone 10) which contained 5'-end truncated *Avin_16040* genes showed no change in its cell morphology. Cloning of 5'- and 3'-end truncated surface layer gene fragments and the expression of N-terminal protein segment (C-terminal or 3'-end gene truncation) are crucial for cell surface display of functional polypeptides (Knobloch *et al.*, 2012). The same study also showed that insertion of functional peptide sequences or single amino acids had no or only slight effect on the formation of S-layer protein sheets. In contrast, the C-terminally truncated S-layer protein has lost its cell surface display function. Typically, the S-layer structural protein consisted of two functional regions. The N-terminal region was involved in the attachment of S-layer subunit to the underlying cell wall (transmembrane) while the middle and C-terminal region was involved in S-layer assembly (Åvall-Jääskeläinen *et al.*, 2008). Our observation matches that of Knobloch *et al.* (2012) showing that the 5' gene segment of Avin_16040 was crucial for its expression as implicated by the formation of filamentous cells. It is also worth mentioning that a 5' gene fragment of as small as 346 bp was able to impose *E. coli* with the shape-shifting effect. Finally, we used AFM to display the cell surface topography of *E. coli* cells which acquired the complete *Avin_16040* gene of 1,368 bp. The images in Figure 6 are in agreement with previous report (Liew *et al.*, 2015) that Avin_16040 over-expresses during its adherence to the rice root. Both Figure 6C and 6D demonstrated elongated *E. coli* cells expressing the *Avin_16040* gene relative to the untransformed cells. However, the over-expression of Avin_16040 were particularly demonstrated by the thickened cell as displayed by the recombinant *E. coli* cells adhering to rice root (Figure 6D). Besides, the root-adhered recombinant cells were enlarged due to its association with rice root. It is interesting to observe that the non-transformed *E. coli* DH5α cells were enlarged and thickened during association with rice root (Figure 6B). Wheeler *et al.* (2015) has provided evidence that peptidoglycan hydrolases play important role in bacterial cell enlargement through hydrolysis and expansion of the peptidoglycan in the bacterial cell wall, exemplified by *Staphylococcus aureus*. Overall, our study provides additional information for use in the future research works involving biosynthesis, assembly and genetic inferences of the S-layer proteins. Besides, our findings may contribute to its design and applications as cell surface display for the various S-layer fusion proteins.

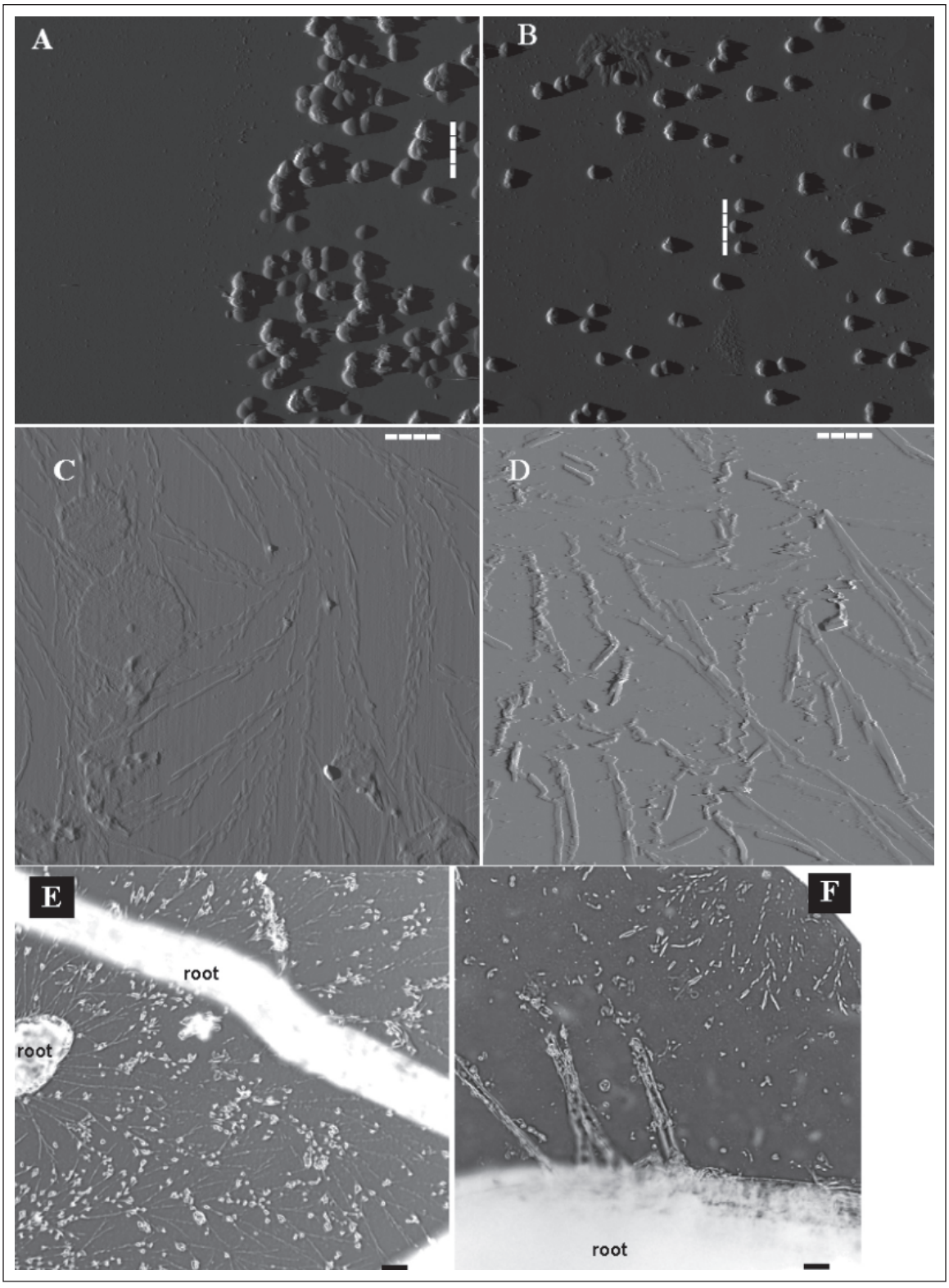
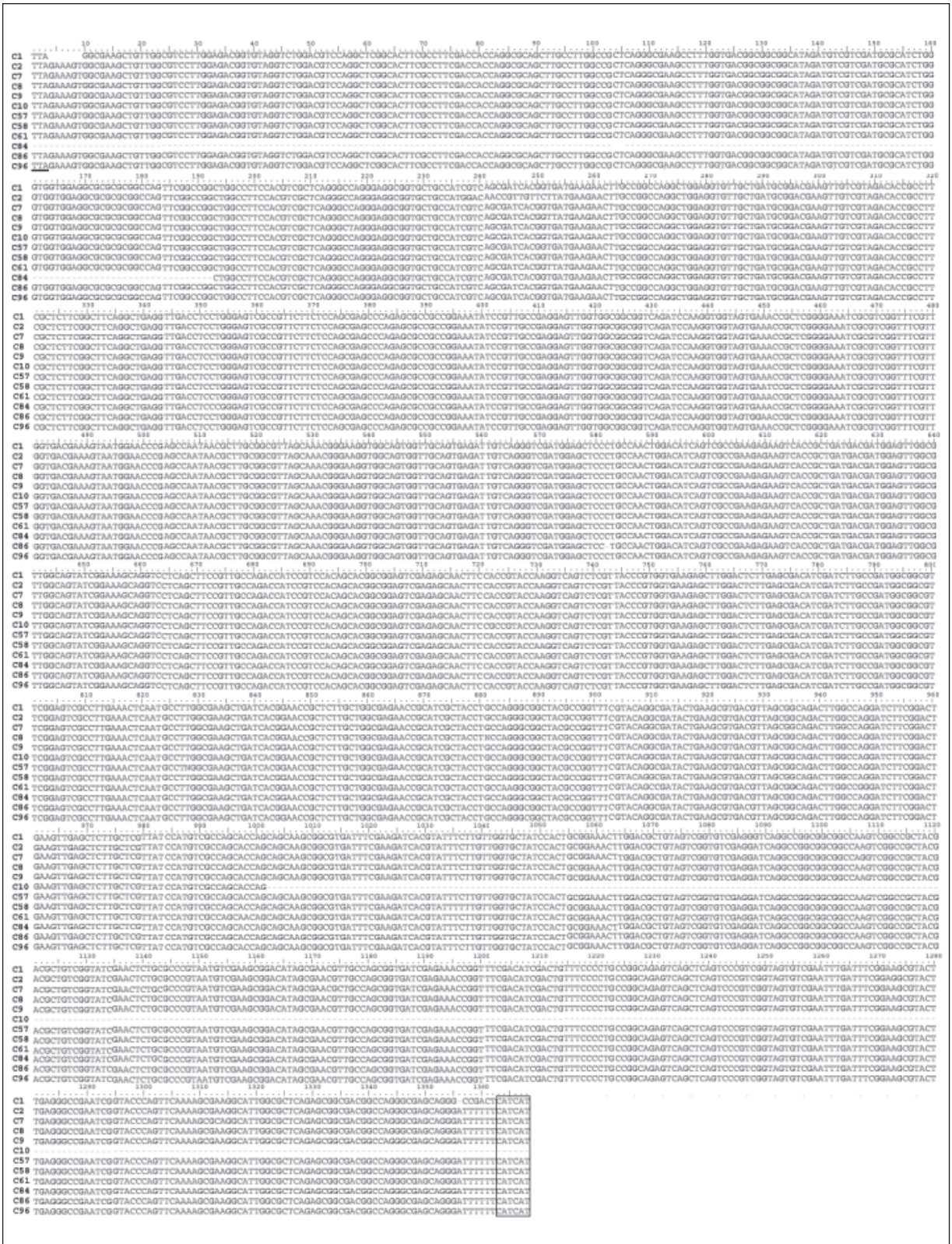


Fig. 3. Observation of *A. vinelandii* Δ*Avin_16040* and ATCC 12837 cells adhered to rice roots. A-D showed the cell surface topographies of Δ*Avin_16040* and ATCC 12837 as displayed by AFM, while E-F showed the compound microscopic observation of root-adhered Δ*Avin_16040* and ATCC 12837 cells observed at 400x magnification. In general, the "non-root-interaction" deletion mutant Δ*Avin_16040* cells (A) displayed bigger cells than the wild type ATCC 12837 cells (B). During root adherence, the mutant cells showed grain-like network and patches (C, E), different from the filament structures of the wild type cells (D, F). The different root-adherence ability of Δ*Avin_16040* and ATCC 12837 cells that was previously reported by Liew *et al* (2015) could be coincided with the observation. A, Δ*Avin_16040* cells (non-root-interaction), B, ATCC 12837 cells (non-root-interaction), C, Δ*Avin_16040* cells adhered to rice root surface, D, ATCC 12837 cells adhered to rice root surface. Scale bar = 10 μm.



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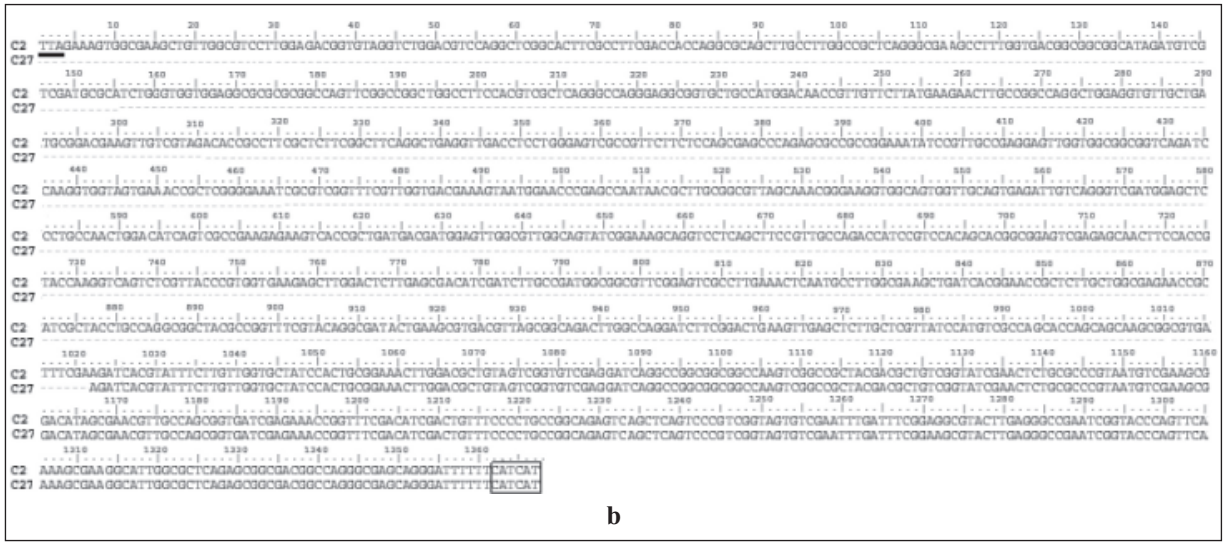

Fig. 4. Multiple alignments of *Avin_16040* gene sequences (gene direction 3' – 5') maintained in the recombinant *E. coli* DH5α clones. Results showed truncated *Avin_16040* genes as indicated by discrepancy in the DNA insert sizes. The start and stop codons are indicated by the black striped boxes and black lines, respectively. a, clones C10 and C84 maintained 3'- and 5'-terminal sequences of *Avin_16040* gene; b, clone C27 maintained a short fragment of *Avin_16040* gene at the 5'-terminal. Complete *Avin_16040* gene consisted of 1,368 nucleotide bases.

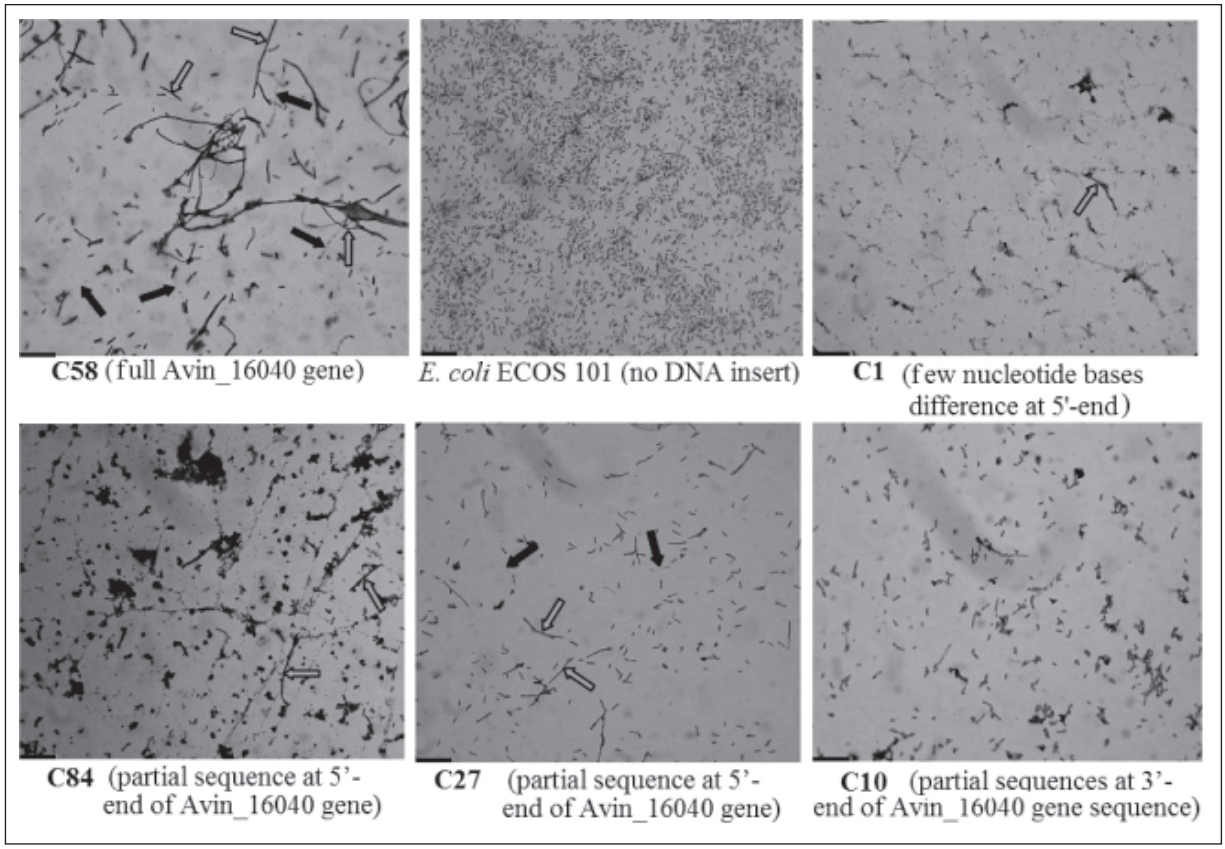

Fig. 5. Compound microscopic images of recombinant clones (Gram stained) at 1000x magnification. The block and black arrows indicate the filamentous cells and "transparent" tube-like structures resulted from the expression of *Avin_16040* gene sequences. The results suggested that the filamentous cell structure was not necessarily caused by complete gene sequence of *Avin_16040*. Instead, some recombinant *E. coli* DH5α clones were found to maintain smaller DNA fragment of the *Avin_16040* gene but still able to acquire the filamentous cell shapes. The lost nucleotide sequences could have been caused by self-deletion or repair mechanism of the heterologous *E. coli* host. Based on the results obtained, the 5'-end of *Avin_16040* gene most possibly consisted of DNA sequence which caused the filamentous cell structures. Scale bar = 10 μm.

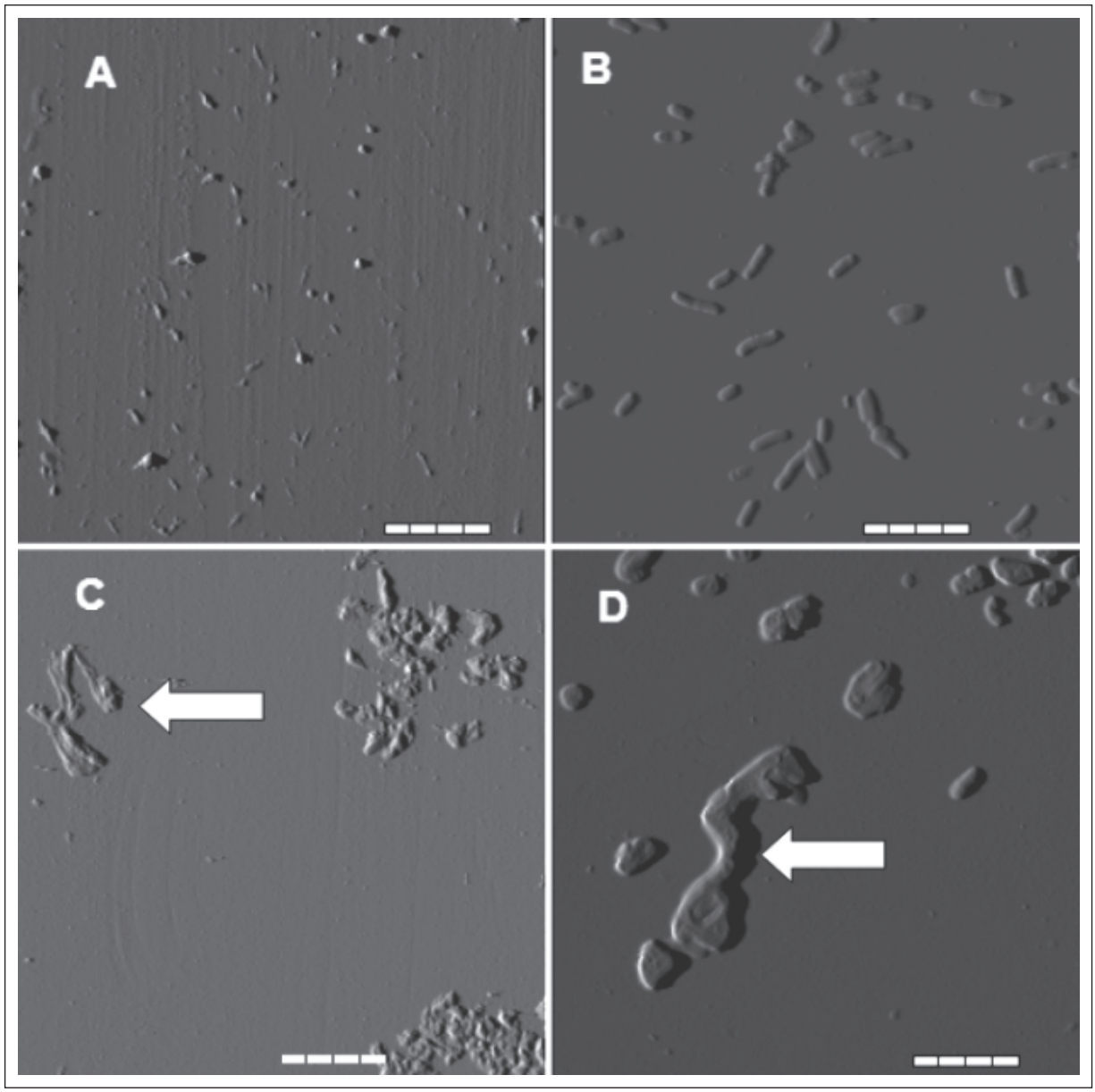

Fig. 6. Cell surface topography of *E. coli* DH5α as displayed by AFM. The images showed recombinant *E. coli* clones transformed with full gene of Avin_16040 (C, D) have developed elongated as well as bigger cells than the untransformed wild type *E. coli* cells (A, B). During root interaction, both the non-transformed *E. coli* cells (B) and *E. coli* cells containing *Avin_16040* gene (D) displayed thicker cell wall appearances. The effect was particularly apparent for the *E. coli* clones containing *Avin_16040* gene. A, non-transformed *E. coli* cells (non-root-interaction), B, non-transformed *E. coli* cells (root-interaction), C, recombinant *E. coli* cells containing *Avin_16040* gene (non-root-interaction), D, recombinant *E. coli* cells containing *Avin_16040* gene (root-interaction). White arrows showed the elongated morphology of *E. coli* cell containing *Avin_16040* gene. Scale bar = 10 μm.

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