Interactomics: Development of an efficient and improved Agrobacterium tumefaciens-mediated transformation method for transient expression of heterologous protein in recalcitrant plant tissues in planta

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Methodology

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Abstract

Background: Since more researches leaned towards a holistic approach of “omics” to understand PPI, interactomics studies pivoting on AP-MS approach are getting popular amongst plant scientists. However, to date, AP-MS approach has yet to be adopted to study plant-pathogen interaction even in model plants, hence, the needs to design and develop an optimised transformation procedure for successful isolation of pathogen-targeted plant proteins.

Methods: Banana plants were grown in soil or hydroponic media for 2-3 months before subjected to transformation procedure. As a bait, m16D10 , a mature parasitism gene isolated from Meloidogyne incognita was transiently expressed in banana root tissues cv. Grand naine (ITC1256) to prey for interacting plant proteins. Subsequently, the plant-pathogen interacting protein complex was “pulled” at the purification step using histidine affinity column, electrophoresed in SDS-PAGE gel and subjected to western blot assay.

Results: We first compared Agrobacterium tumefaciens -mediated transformation quality on root tissues grown in soil and hydroponic media and found that the latter yielded higher area of transformed tissues (99% - 100%). We found hydroponic-grown root tissues were amenable to syringe-based agroinfiltration method and required lesser acclimatisation period compared to the soil-grown root tissues. Our result showed that western blot minimum detection limit of heterologous protein amount in the purified root protein product was at 1:1 ratio, with minimum concentration of 9 mg/mL of purified protein. Using these parameters, western blot assay confirmed the expression of m16D10 gene in transformed plant root tissues and SDS-PAGE gel revealed that we had isolated putative plant proteins interacting with the bait protein.

Conclusion: An improved, efficient, and cost-effective Agrobacterium tumefaciens -mediated transformation protocol was developed for transient expression of heterologous protein in recalcitrant plant tissues, suitable for AP-MS-based plant-pathogen interaction studies. Confirming our hypothesis, we demonstrated that the improved method had successfully “pulled-out” banana root proteins interacting with m16D10 protein using histidine affinity column.

Background

Genotype-phenotype relationships are governed by complex biological systems and cellular networks, primarily mediated by protein-protein interactions (PPI). Since the past 20 years, current information on PPI networks in microbes [1], plants [2] and humans [3] were generated chiefly from Yeast two-hybrid (Y2H) screening approach [4, 5], Co-immunoprecipitation (Co-IP) method [6] and Affinity tagging-purification-mass spectrometry (AP-MS; [7]) method. In combination, these three methods are able to reveal physical connections between proteins and complexes, hence reflecting the complex signalling network underlying biological processes. Of late, interactomics gained a great deal of attention as more

researches leaned towards a holistic approach of 'omics' studies to understand PPI [8], pivoting on AP-MS method [9, 10].

In principle, AP-MS method manipulates antibody-tagging system coupled to a 'bait' protein that is transformed into a cell system. This bait protein will then interact with the 'prey' protein in the cell system in vivo. These interacting units are then isolated as a complex using a purification column that 'pulls' the tagged 'bait' protein. The pulled protein complex is then subjected to mass spectrometry analysis [11–13]. Unlike Y2H, AP-MS requires no prior knowledge on interacting proteins, allowing the discovery of novel insights on protein(s) that is/are interacting with the 'bait' protein [14]. Numerous studies in PPI and interactomics utilised AP-MS method to understand molecular functions of proteins in an organism for different purposes, among others in identifying interacting protein complexes in cancer cells [15], mapping protein interactome in Caenorhabditis elegans [16], studying plant defence mechanisms in Arabidopsis and tobacco against abiotic stresses (reviewed in [17]) and, establishing PPI networks in tricarboxylic acid cycle (TCA) of Arabidopsis [18]. AP-MS was also used to unravel proteins involved in a host-pathogen interaction. This knowledge is crucial to understand the biological mechanism of an infection and serves as a gateway for targeting novel treatments against pathogens [19]. In mammalian cells, AP-MS approach was used to identify host proteins that are targeted by infectious pathogens [19, 20] such as in human-pathogenic bacteria interaction studies [21, 22] and in human-virus interaction studies [7, 23, 24]. However, to date, AP-MS approach has yet to be adopted to study plant-pathogen interaction even in model plants presumably due to the hurdle in delivering parasitism genes into plant tissues, hence, the needs to design an optimised transformation procedure for successful isolation of pathogen-targeted plant proteins.

The most common and extensively developed genetic transformation methods in plants are Agrobacterium-mediated transformation approach and particle bombardment (biolistic) approach [25–27]. Although biolistic was commonly used to express heterologous gene(s) in recalcitrant plant species [27], Agrobacterium-mediated transformation system was preferred due to its simplicity, efficiency and cost effectiveness [28]. However, to date, the latter technique adopted to obtain both stable and transient expression of heterologous gene in plants still revolves around model plants such as Arabidopsis, tobaccos and tomatoes (among others [29–33]) and often, agroinfiltration method was used to induce transient expression of a gene/(s) in these plants [34]. It is noteworthy that, recently, Zhang et al. [35] had developed a transformation method for transient expression of heterologous gene in Arabidopsis thaliana leaf tissues using agroinfiltration method for AP-MS study. However, to the author's knowledge, an Agrobacterium-mediated transformation method for transient heterologous gene expression in non-model plants with recalcitrant tissues such as banana plants [36] is yet to be published.

Recalcitrant plant tissues are hard to be transformed using Agrobacterium tumefaciens-mediated transformation procedure because most of them are resistant to Agrobacterium infection [37]. Therefore, improvements on this transformation procedure such as including a heat or/and hydrolytic enzyme treatment step to the protocol were made to increase transformation efficiency [38]. This additional step is not only cumbersome but increases the experimental time and cost. In this study, we developed a

robust and fairly inexpensive, yet efficient Agrobacterium tumefaciens-mediated transformation system for recalcitrant root tissues suitable for plant-pathogen interaction studies without having to add a heat or hydrolytic enzyme treatment. We hypothesised that the pathogen (bait) protein is expressed in transformed root tissues and interacts with preyed plant proteins, in planta. This interacting PPI complex is “pulled” at the purification step using histidine affinity column, detectable in SDS-PAGE gel.

To model the experiment, we used the mature peptide of 16D10 gene (denoted as m16D10; ~55 bp, ~ 98 kDa) [39], a parasitism gene isolated from an economically important plant-parasitic nematode species, Meloidogyne incognita [40] as the ‘bait’ protein. Coupled to a His tag, this bait protein was then expressed in banana cv. Grand naine (ITC 1256) root tissues to prey for interacting plant proteins in planta. Banana was chosen not only due to its economic significance [41, 42] but also to represent the non-model plants with recalcitrant tissue type. Besides that, since obtaining stable transformed banana cells or tissues requires a long period of time (eight months), which is unnecessary to plant-pathogen interaction studies [43–45], we demonstrated here that our improved experimental protocol can successfully isolate putative banana root proteins interacting with m16D10 within three days post inoculation period.

Results

Cloning of *m16D10* gene into pCAMBIA1304 vector backbone and expression of pCAMBIA1304::CaMV35S::m16D10::mgfp5::GUS::6xHis in *Agrobacterium tumefaciens* (LB4404) cells

m16D10 sequence was successfully cloned into pCAMBIA1304 at the *Nco*I site to make pCAMBIA1304::CaMV35S::m16D10::mgfp5::GUS::6xHis expression cassette, and transformed into *Escherichia coli* (JM109) bacterial system. Detection of ~55 bp fragment in *E. coli* colonies indicates the presence of *m16D10* gene (Figure 1a). PCR results also showed the amplification of bigger-sized bands of ~100 bp and ~150 bp indicating unspecific primer binding. BLASTn analysis revealed that the cloned sequence has 100% similarity with *Meloidogyne incognita*’s *m16D10* parasitism gene sequence in the GenBank (Accession no. DQ087264; Figure 1b), confirming the identity of the cloned sequence. Only plasmids with the correct *m16D10* sequence and orientation (Figure 1c) were transformed into *Agrobacterium tumefaciens* (LB4404) cells.

Agrobacterium tumefaciens-mediated transformation into hydroponic-grown banana plantlets yielded higher portion of transformed root tissues

We first confirmed the expression of pCAMBIA1304::CaMV35S::m16D10::mgfp5::GUS::6xHis in transformed *Agrobacterium tumefaciens* cells by subjecting the cells to GUS histochemical assay. We observed that transformed *A. tumefaciens* cells were of dark blue colouration (Figure 2) when subjected to GUS assay, confirming the expression of *GUS* gene in the transformed cells. Since *GUS* is located downstream of *m16D10* gene in the expression cassette (Figure 3), we interpreted this result to indicate that the whole length of the fusion protein in the expression cassette was also expressed.

We then compared two plant acclimatisation methods to obtain the knowledge on which method is better in producing increased number of transformed banana root tissues for subsequent analyses. Here, we found the number of roots of banana plants acclimatised in hydroponic system was higher and healthier compared to the ones acclimatised in the soil (data not shown). Furthermore, we also observed that, it took two months for the roots of hydroponic-grown plants to reach 0.4 cm in diameter and ready for *A. tumefaciens*-mediated transformation procedure while the root system of soil-grown plants remained inferior in characteristics (i.e. small in numbers and low in diameter) even after three months. Therefore, the acclimatisation period in soil was terminated at the third month. In addition, the roots grown in hydroponic media were softer in texture, rendering amenability for agroinfiltration procedure compared to that of grown in soil.

We conducted a GUS histochemical assay on root tissues from soil-grown plants and hydroponic-grown plants post-transformation and observed that the area of transformed root tissues in the former was lower (Figure 4) compared to that of the latter (Figure 5). When quantification was made on the photographed image using ImageJ software, the average percentage of transformed root area (GUS-stained) of root tissues grown in soil at 1-day post inoculation (dpi) was 7% (n= 3 root fragments) and 63% for root tissues grown in hydroponic culture (n = 2 root fragments) (Figure 6a, Supplementary Table 1S). Since the percentage of transformed root area was higher in the hydroponic-grown root tissues, we further extended the transformation period to 3-, 5- and 15-dpi and found that the average percentage of transformed root area was 97%, 100% and none, respectively (Figure 6b, Supplementary Table 1S). No transformed area detected at 15-dpi confirmed the transient expression nature of the transformed protein. It is noteworthy that, although the root tissues were 100% transformed after 5-dpi, our observation showed that the root tissues started to wilt and blackened at this point onwards (Figure 5). On the contrary, the transformed root tissues at 3-dpi remained healthy while maintaining high number of transformed areas, hence, chosen as the best time-point for subsequent analysis. Note that control treatment of transformed roots with untransformed *A. tumefaciens* culture showed no expression after GUS assay (Figures 4 and 5).

Western blot minimum detection limit of m16D10 protein amount in the purified root protein product is 15 mg

Since, there were no available literatures outlining the amount of heterologous protein detectable in plant tissue extract where the protein was transiently expressed in, we carried out an experiment to determine the minimum detection limit of purified banana root protein samples containing m16D10 protein in a western blot assay by spiking the total root protein with the total protein of transformed *Agrobacterium tumefaciens* cells. Four different amounts of total crude transformed *A. tumefaciens* protein were used i.e. 3.75 mg, 7.5 mg, 11.25 mg, and 15 mg. In this experiment, we only successfully detect the presence of ~98kDa protein band in western blot assay of purified protein when 15 mg transformed *A. tumefaciens* protein were spiked to 15 mg total crude banana root protein (Figure 7). Note that the size of detected protein band corresponds to the size of m16D10 protein. Our result revealed that the ratio of *A. tumefaciens* protein amount to banana root protein amount detectable in western blot assay of purified

banana root protein was 1:1 (Supplementary Table 2S). On the other hand, other amounts of *A. tumefaciens* protein (3.75 -11.25 mg) yielded no detection signal in western blot assay (data not shown). We also found that the minimum limit of protein concentration of purified protein product (transformed *A. tumefaciens* protein + banana root protein) sufficient to yield signal in western blot assay was 9 mg/mL.

Successful isolation of banana root proteins interacting with m16D10 *in planta* using hydroponic-grown root tissues

Total crude root protein was isolated from root fragments containing transformed root cells from both soil- and hydroponic-grown root tissues. We found protein isolated from the former can neither be detected on SDS-PAGE nor western blot assay compared to the latter. When the total crude protein from soil-grown transformed root tissues was purified, the concentrations obtained were within the range of 0.3 to 0.4 mg/mL, suggesting a low number of transformed cells. This was in stark contrary to the concentration obtained from purified protein of hydroponic-grown root tissues, that was within the range of 8 -10 mg/mL. We therefore subjected the total crude root protein sample isolated from hydroponic-grown root fragments to purification procedure. SDS-PAGE gel of purified transformed banana root tissues revealed the presence of protein bands of different sizes (Figure 8a) suggesting successful isolation of putative banana root proteins interacting with transiently expressed m16D10 parasitism protein (protein identification result will be published elsewhere). To confirm the interaction of these plant proteins with m16D10, the SDS-PAGE gel was subjected to western blot assay to detect the presence of m16D10 protein in the purified protein. Western blot assay using anti-His antibody revealed a detection of ~98 kDa protein band corresponding to m16D10 protein on the nitrocellulose film (Figure 8b) confirming the presence of transiently expressed heterologous nematode protein.

Discussion

Understanding protein-protein interaction (PPI) in plant-pathogen communication is crucial for the development of an effective pathogen management programme. Many scientists studying human-pathogen interaction had opted for the interactomics approach to identify host proteins targeted by infectious pathogens. Often, interactomics revolves around the usage of affinity tagging-purification-mass spectrometry (AP-MS) method using tagged ‘bait’ protein to isolate the ‘preyed’ host proteins as a strategy. In plants, although AP-MS method has been used to study plant defence mechanisms against abiotic stresses (reviewed in [17]) and establish PPI networks in tricarboxylic acid cycle (TCA) [18], this method has yet to be adopted for plant-pathogen interaction studies, not even in model plants. Here, we developed an improved Agrobacterium-mediated transformation protocol for recalcitrant plant tissues using the mature peptide of 16D10 gene (denoted as m16D10), a parasitism gene isolated from Meloidogyne incognita, and banana cv. Grand naine (ITC 1256) root tissues as our experimental model. Since AP-MS method requires high amount of starting material to enable successful purification step [46], we first obtained the information on which plant-growing method could produce increased number of transformed banana root tissues.

Our observation showed that the number and quality of root system grown in hydroponic media were superior compared to that of grown in soil. The higher number of roots in hydroponic-grown plants may be attributed to the higher amount of nutrient uptake [47] of hydroponic plants. Besides, Surendran et al. [48] reported that hydroponically grown plants demonstrated higher antioxidant activity, enzyme activity, antimicrobial activity, and higher amount of organic acids compared to plants grown in soil rendering them to be healthier than that of grown in soil. In addition, as opposed to three-month acclimatisation period, we found that hydroponic-grown root tissues took only two months to reach the ready stage for Agrobacterium tumefaciens- mediated transformation procedure. When GUS histochemical assay was conducted on root tissues from soil-grown plants and hydroponic-grown plants post-transformation procedure, we observed that the area of transformed root tissues in the former was lower (Fig. 4) compared to that of the latter (Fig. 5). Our result on quantification analysis of the average percentage of transformed root area (GUS-stained) of the transformed root tissues using ImageJ software [68] corroborates our observation (Fig. 6; Supplementary Table 1S). We relate this result to the agroinfiltration procedures adopted on root tissues of two differently grown banana plants.

Syringe-based agroinfiltration method is an established method for transient expression in plants (reviewed in [49]). However, this method was primarily optimised in model plants such as Arabidopsis thaliana [50] and Nicotiana benthamiana [51], and quite recently in other non-model plants such as strawberry [52], soybean [53] and tomato [54]. To date, this method has yet to be adopted on banana tissues. Despite being widely reported (reviewed in [55]), we were not able to adopt syringe-based agroinfiltration method on the soil-grown banana root tissues because of the hardiness of the root epidermis, rendering rejection of transformation solution injected into the root tissues. Therefore, scarring was the next best option to agro-infiltrate the root tissues. It is noteworthy that Chen et al. [56] had adopted similar transformation technique on soybean seeds. In contrast to that, the root tissues grown in hydroponic media were softer, making it amenable for syringe-based agroinfiltration procedure, hence, increasing the number of transformed cells. Since the percentage of transformed root area was higher in the hydroponic-grown root tissues at 24-hour post inoculation (hpi) compared to that of grown in soil, we further extended the transformation period of the former to 3- days post inoculation (dpi), 5- and 15-dpi to obtain the best time-point to harvest the plant. Our observation showed that, GUS-expression was not detectable at 15-dpi and the average percentage of transformed root area was 97% and 100% at 3- and 5-dpi, respectively (Fig. 6b, Supplementary Table 1S). Although the percentage of transformed area was higher at 5-dpi, we suggest for heterologous gene expression to be halted at 3-dpi (the longest). At this time point, transformed plant tissues showed comparable percentage of transformed area with 5-dpi without compromising the root integrity (wilting and blackening of root tissues). Note that no GUS expression observed in transformed root fragments at 15-dpi indicates the transient manner of the heterologous gene expression. To meet our objective that was to capture bait-prey protein complex from the transformed root tissues, we subjected the isolated root proteins to protein purification step using nickel His Gravitrap™ affinity column and western blot assay. This was the point where we struggled the most in this study.

Obtaining enough concentration of purified protein yielding sufficient amount of detectable transiently expressed protein in western blot assay was an uphill task. Ghosh et al. [60] in their review highlighted the importance of sample preparation and the amount of protein loaded onto gels for subsequent western blotting procedure. In order to detect a protein signal in western blot assay using anti-GUS primary antibody and anti-mouse IgG- alkaline phosphatase secondary antibody, a sufficient amount of purified protein must be loaded onto SDS-PAGE gels. Since the information on the amount of heterologous protein in a purified plant protein sample detectable in a western blot assay was, as yet, unavailable, we carried out an experiment to determine the minimum western blot's detection limit of purified banana root protein samples containing transiently expressed m16D10 protein. Here, by detecting the ~ 98 kDa m16D10 protein band, we demonstrated that the detection of protein signal in a western blot assay was only possible if the amount of heterologous protein to the amount of banana root protein was at 1:1 ratio (Fig. 7; Supplementary Table 2S). Lower amount of m16D10 protein used (3.75–11.25 mg) in 15 mg banana root protein did not yield any signal in the said assay, suggesting high number of transformed cells is required in the transformed root tissues for successful detection of heterologous protein expression. We also found that the minimum limit of protein concentration obtained after purification procedure sufficient to yield signal in western blot assay was 9 mg/mL. Here, we concur with Ghosh et al. [60] that an optimised amount of protein is crucial for efficient signal detection in a western blot assay.

In this study, we have successfully isolated banana root proteins (~ 16 to 175 kDa) potentially interacting with m16D10 parasitism protein, in planta, using the optimised parameters (Fig. 8). We confirmed that the interaction was indeed with m16D10 parasitism protein by detecting the presence of ~ 98 kDa protein band in western blot assay of purified protein sample isolated from transformed banana root tissues. It is important to highlight here that a smaller protein band of ~ 80 kDa (Fig. 8b) was also observed on the nitrocellulose membrane. Since the band was not present on the SDS-PAGE gel used in the blotting assay (Fig. 8a), we hypothesised that this band is an artefact resulting from non-specific binding of antibodies used in the assay. Non-specific binding of commercialised antibodies in western blot assays has been discussed in [57, 58]. In order to minimise the un-specificity of antibody binding, we suggest for using lower concentration of antibody and varying the length of incubation period during western blot assay as proposed in [59]. To further confirm the identity of the proteins isolated from this study and specificity of the interaction, protein bands obtained in SDS-PAGE gel should be subjected to mass spectrometry analysis. Note that unspecific protein-protein interaction using AP-MS approach was highlighted in [7]. Therefore, the interactions obtained from such studies should further be validated using other specific methods such as Y2H, co-immunoprecipitataion (Co-IP) and bimolecular fluorescence complementation (BiFC) [60, 61].

Conclusion

In conclusion, we had successfully developed an efficient, improved, and cost-effective Agrobacterium tumefaciens-mediated transformation protocol for transient heterologous protein expression in recalcitrant plant tissues, suitable for protein-protein interaction (PPI) studies. Using a mature Meloidogyne incognita 16D10 parasitism protein (m16D10) and banana root tissues as our experimental

model, we demonstrated here that syringe-based agroinfiltration procedure on hydroponic-grown banana root tissues carried out at 3-day post inoculation (dpi) yielded higher portion of transformed root tissues, suitable for AP-MS studies. Confirming our hypothesis, we demonstrated that the improved method had successfully pulled out banana root proteins interacting with m16D10 protein using histidine affinity column, observed on SDS-PAGE gel. We notion that not only our improved-method is suitable for plant-pathogen interaction studies, this method also serves as a strong foundation for others to embark on any protein-protein interaction studies, in planta.

Methods

Plant materials

Banana cv. Grand naine (ITC 1256) plantlets were obtained from the International Transit Centre (ITC) of *Musa* collection at Katholieke Universitiet Leuven, Belgium. The plantlets were propagated and maintained in banana multiplication media (Plantigen™ HIMEDIA, India) according to the manufacturer's protocol. Subsequently, the plantlets were transferred onto rooting media (Plantigen™ HIMEDIA, India) for a month until reaching a five-leaf-stage prior to transplantation into either soil or hydroponic culture media.

Cloning of mature *16D10* gene sequence into pCAMBIA1304 expression vector

We synthesised *16D10* gene sequence published by [39] in pUC57 vector (NextGene, Malaysia). Mature peptide (denoted as m16D10) was used in this experiment. *m16D10* gene was cloned to pCAMBIA1304 expression vector with 35S promoter driving its expression. For cloning purposes, the primer pair m16D10F 5' CATCCATGGGCAAAAAGCCTAG 3'/ m16D10R 5' GACCTCCTTTATTAAGGTACCGAT 3' was designed to include *Nco*I restriction enzyme (RE) site (sequence underlined) to create sticky ends to the amplified *m16D10* fragment. PCR was conducted in a thermocycler (peqSTAR, USA) with amplification profile consisted of an initial denaturation step at 94 °C for 3 minutes, followed by 35 cycles of a denaturation step at 94 °C for 1 min, an annealing step at 52 °C for 1 min, an elongation step at 72 °C for 44 sec, and a final extension step at 72 °C for 5 min. A total of 5 µL PCR product were then subjected to agarose gel electrophoresis (AGE) analysis. PCR product with the correct expected size (~55 bp) was purified using QIAquick gel extraction kit (Qiagen, USA) according to the manufacturer's protocol. Both pCAMBIA1304 vector and the purified *m16D10 gene* were digested with *Nco*I enzyme and purified again prior to cloning. Both purified products were ligated using T4 DNA ligase (NEB, UK), producing pCAMBIA1304::CaMV35S::m16D10::mgfp5::GUS::6xHis expression cassette (Figure 3). We then propagated the plasmid in *Escherichia coli* (JM109) bacterial system.

Plasmid isolation from *Escherichia coli* and sequence analysis of cloned m16D10 fragment

Plasmid DNA was isolated from *Escherichia coli* cell lysate to detect the presence of m16D10 cloned fragment via PCR using the same primer pair. We confirmed the nucleotide sequence of the construct via one pass sequencing procedure using CaMV35S promoter region primer 5' CTTTATTGTGAAGATAGTGGA

3’. Sequencing result was analysed using Chromas (Technelysium Pty Ltd, Australia) and subjected to BLASTn analysis at the GenBank [62]. Only clones with correct sequence and orientation were transformed into *Agrobacterium tumefaciens* strain LBA4404*.*

Transformation of pCAMBIA1304::CaMV35S::m16D10::mgfp5::GUS::6xHis into *A. tumefaciens* LBA4404 cells and preparation of transformation solution

pCAMBIA1304::CaMV35S::m16D10::mgfp5::GUS::6xHis was transformed into *A. tumefaciens* LBA4404 competent cells using a freeze-thaw method as described in [63]. Positive colonies harbouring the desired construct were selected using Kanamycin antibiotic. Plasmids were isolated from the surviving colonies and sent for sequencing to confirm the sequence and orientation of the construct. We also confirmed the expression of plasmid construct in *A. tumefaciens* clones using β-glucuronidase (GUS) histochemical assay.

GUS assay was conducted on putatively transformed *A. tumefaciens* cells as described in [64]. Briefly, the bacterial cells were incubated in a GUS solution (0.1 M $NaPO_4$, 10 mM EDTA pH 8, 0.1% Triton X-100, 1 mM $K_3Fe(CN)_6$, 2 mM X-Glucuronide) at 37 °C for 16 hours. *GUS* gene, expressing β-Glucuronidase (GUS) enzyme hydrolyses X-Glucuronide substrate at 37 °C. The product of the hydrolysation i.e. Glucuronide, dimerised in the presence of oxygen to form an insoluble blue precipitate of chloro-bromoindigo [65], yielding blue-stained bacterial cells that are visible to the naked eye. The observation was photographed. We expected that *m16D10* gene was also expressed in this construct since *GUS* gene is located downstream of *m16D10* in the expression cassette and the expression was driven by the same promoter (Figure 3). Only clones with expressed cassettes were subjected to *in planta* root transformation procedure.

Transformation solution was prepared according to [66]. Briefly, 200 µL of inoculated *A. tumefaciens* glycerol stock were cultured overnight in Luria-Bertani (LB) media (LB 20 g/L concentration, 50 µg/mL Kanamycin) at 28°C with 200 rpm shaking in a dry incubator. The culture was transferred into sterile 50 mL Falcon tubes once the $O.D_{600}$ value reached 1.0 and centrifuged at 4000 x *g*, 4 °C for 10 minutes to pellet the cells. The pelleted cells were then diluted with sterile LB media (pH 6) until $O.D_{600}$ value reached 0.3. This transformation solution was then used to transform *A. tumefaciens* harbouring pCAMBIA1304::CaMV35S::m16D10::mgfp5::GUS::6xHis into banana root tissues, *in planta*.

Transformation of soil-grown banana root tissues with *A. tumefaciens* (LBA 4404) cells harbouring pCAMBIA1304::CaMV35S::m16D10::mgfp5::GUS::6xHis

We conducted plant root transformation procedure using single inoculation protocol adopted from [67] with slight modifications. Instead of using nematode individuals, we used transformed *A. tumefaciens* cells harbouring pCAMBIA1304::CaMV35S::m16D10::mgfp5::GUS::6xHis as an inoculum. Soil mixture used in this study was first autoclaved at 121 °C for 15 minutes. A batch of 10 banana tissue cultured plantlets were transplanted into 1L pots containing a mixture of soil (black soil, burnt soil, compost, clay and coconut fibre) and sand at 3:1 ratio (Glorious Nursery, Malaysia). These plantlets were left to

acclimatise in a growth room at 30 ± 2 °C with 12 hours photoperiod for three months. Subsequently, root tissues with a diameter ranging from 0.4 cm to 0.6 cm were selected for *A. tumefaciens*-mediated transformation assay. Three plants were treated with transformed *A. tumefaciens* cells while another three served as negative control plants, were inoculated with non-transformed *A. tumefaciens* cells. Three root strands were selected per plant for the treatment. Selected roots were first washed with distilled water, tapped on a C -fold towel and let dry for a few minutes. The roots were then scarred along its length using a sterile scalpel and placed across a 90 x 15 mm petri dish. We inoculated 200 µL transformation solution containing *A. tumefaciens* cells onto each of the scarred root fragment. The treated root fragments were then covered in soil and were harvested 24 hours post-inoculation (hpi). A fragment of the harvested roots in each treatment was subjected to β-glucuronidase (GUS) histochemical assay to confirm the presence of transformed cells in the root tissues, while others were subjected to protein extraction procedure.

Transformation of hydroponic- grown banana root tissues with *A. tumefaciens* (LBA 4404) cells harbouring pCAMBIA1304::CaMV35S::m16D10::mgfp5::GUS::6xHis

A batch of eight plantlets were transplanted into an in-house-designed hydroponic system made of used 1L mineral water bottles (Figure 9a). The plantlets were acclimatised in commercially available hydroponic media (Kazz Hidroponik, Malaysia), in a growth room at 30 ± 2 °C with 12 hours photoperiod for two months. During the acclimatisation period, the medium was replaced once a week while the container was washed every two weeks with 6% bleach to prevent algal or microbial contaminations.

Similar to *A. tumefaciens*-mediated transformation carried out on banana roots grown in soil, root strands with a diameter ranging from 0.4 cm to 0.6 cm were selected to be transformed. However, for hydroponic-grown root tissues, treatment was conducted using syringe-based agroinfiltration technique (Figure 9b). Four plants were treated with 2 mL transformation solution containing transformed *A. tumefaciens* cells using a sterile 3 cc/mL Terumo® syringe and needle while another four served as negative control plants, inoculated with untransformed *A. tumefaciens* cells. Treatment was terminated at four time points, i.e 1- day post inoculation (dpi), 3-, 5-, and 15- dpi. Two fragments of harvested root tissues of each time point were subjected to GUS assay, while others were subjected to protein extraction procedure.

GUS histochemical assay on transformed banana root tissues and estimation of transformed root area

GUS histochemical assay was carried out as described above on harvested root fragments to gauge the percentage of transformed areas on the root tissues. Briefly, harvested root tissues were submerged in GUS staining solution [(0.1 M $NaPO_4$, 10 mM EDTA pH 8, 0.1% Triton X-100, 1 mM $K_3Fe(CN)_6$, 2 mM X-Glucuronide] in a 15 mL Falcon tube and later incubated at 37°C for 16 hours. Images were photographed. Estimation of transformed root area was made on these photographed images using ImageJ software [68] based on the number of pixels that made the image up. The percentage (%) of transformed root area for each root fragment was defined as [(the total number of pixels of GUS-stained

root area / the total number of pixels of the whole root fragment) x 100], with final values obtained rounded. For this analysis, triplicates were used for root tissues grown in soil while duplicates were used for those grown in hydroponic culture for each time-point.

Total crude protein isolation from *A. tumefaciens* (LB4404) cells

Total crude protein was isolated from *A. tumefaciens* (LB4404) as described in [69] with slight modifications. Briefly, 200 µL *A. tumefaciens* cells were cultured in 1 L Luria-Bertani (LB) media containing 50 µg/mL Kanamycin at 28°C with 200 rpm shaking overnight in an incubator (N-Biotek, South Korea), until the value of $O.D_{600}$ reached 1.0. The culture was then transferred into four sterile 250 mL Nalgene bottles and centrifuged at 4000 x *g,* 4 °C, for 10 minutes. Subsequently, the supernatants were discarded, and the pellets were stored at -80°C. To harvest the protein, the pellets were subjected to four cycles of freeze-thaw procedure which includes freezing at - 80°C for 10 minutes and thawing at 0 °C (ice-water bath) for 20 minutes. The pellets were then resuspended in a resuspension buffer [10% Glycerol, 25 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.1% Tween 20 and 1 tablet /10ml EDTA-free protease inhibitor cocktail (Roche, Germany)] as described in [70]. The resuspended solution was transferred into a fresh 15 mL Falcon tube and incubated in ice for 60 minutes. Soluble protein sample was collected by subjecting the sample to a centrifugation step at 6000 x *g*, 4 °C for 15 minutes. The isolated total protein was either used as a positive control to detect expressed protein or used in the 'spiking' experiment.

Total crude protein isolation from banana root tissues and protein purification using nickel His Gravitrap^{TM} affinity column

Total crude protein was isolated from harvested root fragments according to [71] with slight modifications. Briefly, the harvested root tissues were ground into fine white powder in the presence of liquid nitrogen using a sterile mortar and pestle pair. Approximately 100 - 150 mg banana root powder were mixed with 5 mL of ice-cold extraction buffer [25 mM Tris-HCl pH 7.6, 15 mM $MgCl_2$, 150 mM NaCl, 0.1% Triton X, 0.1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 1 µM E-64, 5% Ethylene glycol, 60 mM β-glycerophosphate and 1 tablet/10mL EDTA-free protease inhibitor cocktail (Roche, Germany)] in a sterile 15 mL Falcon tube. Subsequently, 0.1% (vol/vol) Benzonase were added to the mixture and incubated at 4 °C for 30 minutes. Subsequently, the tube was centrifuged for 10 minutes, 6500 x *g*, 4 °C, and the resulting supernatant transferred into sterile 2 mL microcentrifuge tubes. The samples were later centrifuged at the maximum speed (6500 x *g*), 4 °C for 30 minutes or until a clear supernatant was obtained. The supernatant was then transferred into fresh Falcon tubes and either subjected to protein purification step or stored at -80 °C. To analyse the quality of extracted crude protein, 10 µl of the extract were subjected to SDS-PAGE and western blot analysis.

Protein purification step was carried out by subjecting 30 mL total crude root protein to nickel His Gravitrap^{TM} purification column (GE Healthcare, Sweden) according to the manufacturer's protocol. Purified protein was eluted with an elution buffer (20 mM Sodium phosphate, 500 mM NaCl, 500 mM

Imidazole), and quantified using Bradford assay. Purified sample was subjected to SDS-PAGE and western blot analysis.

SDS-PAGE and western blot assay

Total crude root protein extract or nickel-purified root protein (1 µg/µl) was separated by SDS-PAGE at 180V for 1hr (Bio-rad, USA), and stained with Coomassie blue solution [50% (v/v) methanol, 0.05% (v/v) Coomassie Brilliant Blue R250, 10% (v/v) acetic acid]. For western blot analysis, the electrophoresed protein sample on SDS-PAGE gel were transferred onto a nitrocellulose membrane (GE Healthcare, USA). The membrane was blocked with skimmed milk (2.5 g of skim milk in 50 mL of 1X TBS) at 4 °C overnight then blotted with 2 µg anti-GUS primary antibody (ID# ab188492; Abcam, USA) and 2 µg anti-mouse IgG-alkaline phosphatase, produced in goat secondary antibody (Cat. #SLBB9232, Sigma-Aldrich, USA). Protein bands on SDS-PAGE and membrane were photographed.

Determination of minimum amount of m16D10 protein signal in the purified root protein product detectable in western blot assay

An experiment referred to as 'spiking' experiment was conducted to determine the minimum amount of m16D10 protein detectable in western blot assay after the total root protein purification step. To mimic the expression of pCAMBIA1304::CaMV35S::m16D10::mgfp5::GUS::6xHis in transformed root tissues, we spiked 15 mg total crude root protein sample with different amounts of m16D10 protein that were expressed in *A. tumefaciens*. m16D10 protein amounts used in this experiment were 3.75 mg, 7.5 mg, 11.25 mg, and 15 mg. This made up the ratio of bacterial protein: crude plant root protein amount of 0.25:1, 0.5:1, 0.75:1 and 1:1, respectively. The protein mixture was then subjected to a purification step, quantification step, and western blot assay as described above.

Supplemental Information Note

Additional files

Additional file 1. Supplementary Table 1S

Abbreviations

CaMV: cauliflower mosaic virus; dpi: day-postinoculation; GUS: β-glucuronidase; His: histidine; hpi: hour-post inoculation; kDa: kilo Dalton; m16D10: mature peptide of 16D10 gene isolated from Meloidogyne incognita; mgfp5: modified GFP protein with an endoplasmic reticulum targeting sequence; SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis; PCR: Polymerase chain reaction.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

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Data curation: NDA, NHM

Formal Analysis: NDA, NHM, AA

Funding acquisition: AA

Investigation: NDA, NHM, AA

Methodology: NDA, NHM, AA

Project administration: AA

Resources: AA

Supervision: AA

Validation: NHM, AA

Visualisation: NDA, NHM

Writing-original draft: NDA, NHM

Writing- review & editing: NHM, AA

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Figures

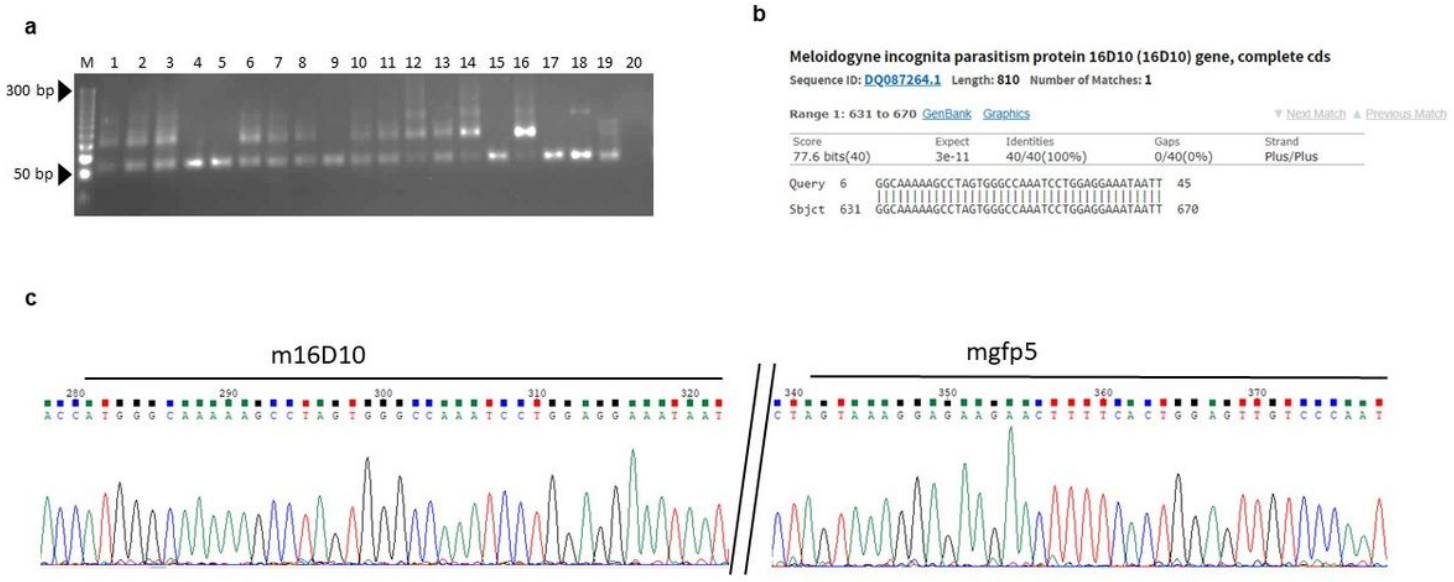

Figure 1

Cloning results of m16D10 gene into pCAMBIA1304 vector. Successful cloning of m16D10 gene into pCAMBIA1304::CaMV35S::m16D10::mgfp5::GUS::6xHis expression cassette confirmed by (a) the detection of ~55 bp PCR band in Escherichia coli cell lysate corresponding to the size of m16D10 gene sequence, (b) BLASTn analysis showing 100% similarity of cloned gene with Meloidogyne incognita's 16D10 parasitism gene sequence in the GenBank (Accession no. DQ07264), and (c) abridged chromatograms showing correct sequence and orientation of m16D10 fused with mgfp5 gene.

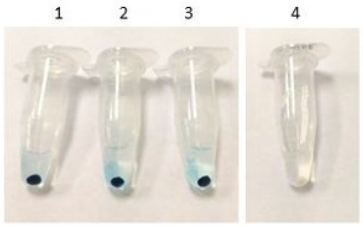

Figure 2

Expression of GUS in Agrobacterium tumefaciens cells. GUS histochemical assay conducted on Agrobacterium tumefaciens cells harbouring pCAMBIA1304::m16D10::mgfp5::GUS::6xHis showed dark blue colouration (tubes 1-3), confirming the expression of GUS gene in the expression vector. Since GUS gene is located downstream of m16D10 in the expression cassette, this result indicates that the whole length of fusion protein in the expression cassette was also expressed. Note that the colour of non-transformed A. tumefaciens cells (negative control) was not changed when subjected to the same assay.

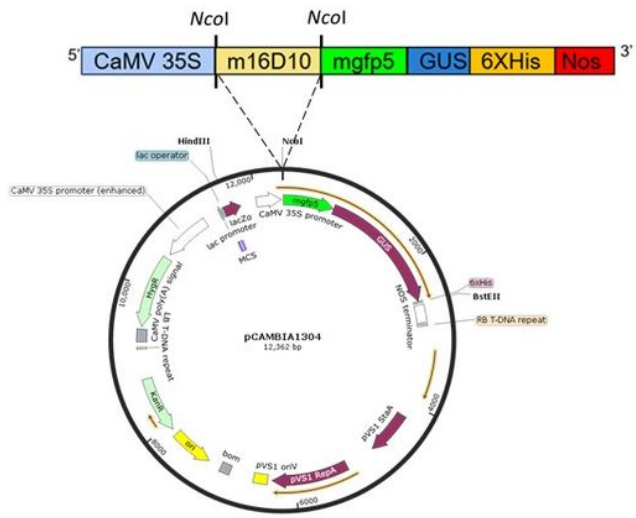

Figure 3

The map of pCAMBIA1304 plasmid vector. The gene encoding the mature 16D10 parasitism protein (denoted as m16D10) was cloned in the multiple cloning site (MCS) of the expression cassette and fused to mgfp5 and GUS genes that served as reporter proteins, and 6xHis serving as affinity tag to facilitate protein purification. The expression cassette is magnified for clarity.

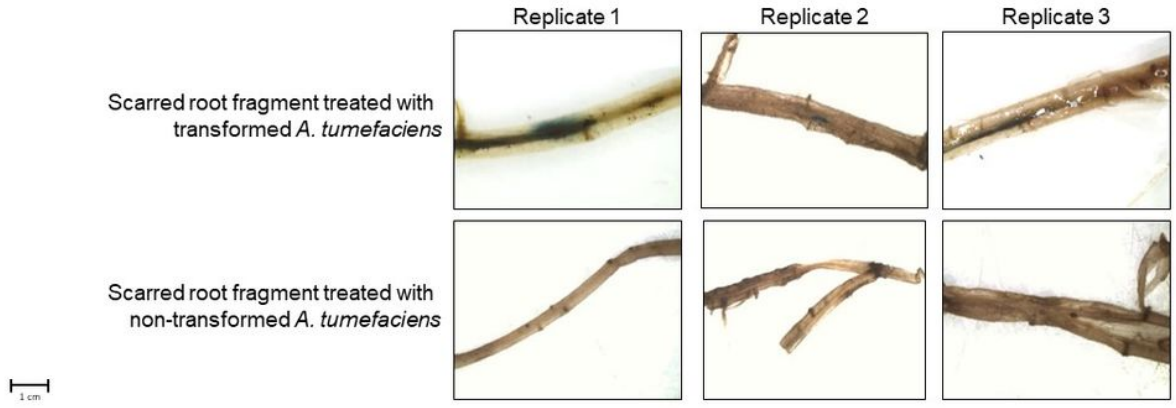

Figure 4

GUS histochemical assay on transformed root fragments harvested from soil-grown banana plantlets. Patches of dark blue areas were observed along the length of transformed root fragment indicating the expression of GUS gene in these root tissues (n=3) as opposed to none in root tissues transformed with non-transformed Agrobacterium tumefaciens cells (n=3). A. tumefaciens-mediated transformation procedure was conducted on banana roots by scarring the root tissues after three months of acclimatisation period in the soil following Sayed Abdul Rahman et al. [67]with minor modifications. Experiment was terminated 24-hour–post-inoculation (hpi).

Figure 5

GUS histochemical assay on transformed root fragments harvested from hydroponic-grown banana plantlets. Transformation procedure was conducted at four time points i.e. 1-day post inoculation (dpi), 3- , 5- , and 15-dpi. Dark blue area was observed along the length of each transformed root fragment at 1- , 3- , and 5-dpi indicating the expression of GUS gene in these root tissues (n=2). However, GUS expression was not observed in transformed root tissues at 15-dpi indicating the transient manner of the gene expression. Dark blue colouration was not observed on negative control root fragments. Treated banana root fragments were agroinfiltrated with A. tumefaciens cells harbouring pCAMBIA1304::CaMV35S::m16D10::mgfp5::GUS::6xHis using a sterile 3 cc/mL Terumo® syringe and needle while negative control root tissues were transformed with non-transformed A. tumefaciens cells.

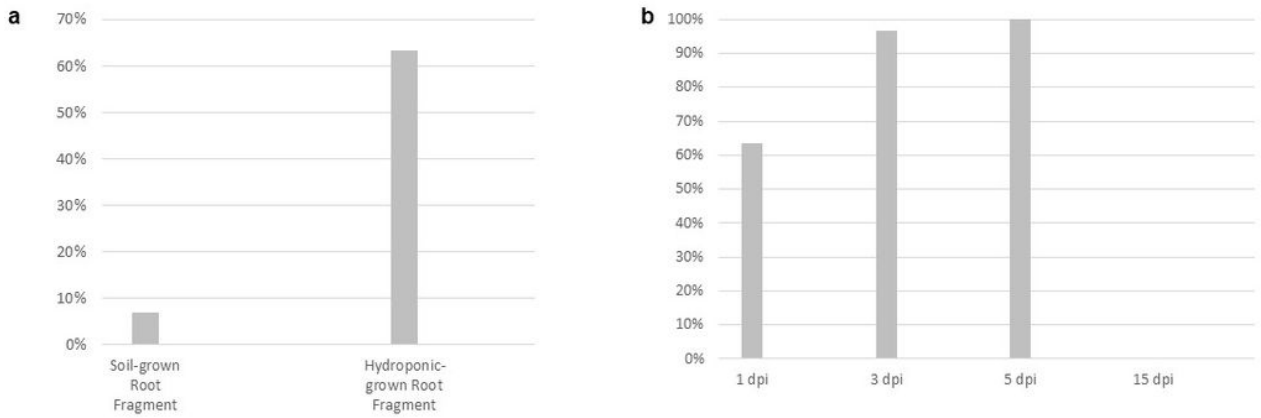

Figure 6

Average GUS-stained area in transformed root tissues. Quantification using ImageJ software [68] on photographed images of transformed root fragments revealed (a) the average percentage of GUS-stained area was higher in transformed root fragments harvested from banana plants grown in hydroponic culture (63%) compared to only 7% in transformed root fragments harvested from soil-grown banana plants at 1-day post inoculation (dpi), and (b) the average percentage of GUS-stained root area in transformed root fragment was the highest at 5-dpi with 100% transformed area, followed by 3- and 1-dpi with 97% and 63%, respectively when Agrobacterium-mediated transformation was conducted on hydroponic-grown banana plants at four time points i.e. 1-, 3- , 5-, and 15- dpi. No transformed area was detected by the software at 15-dpi. Average transformed area was defined as [(the total number of pixels of GUS-stained root area / the total number of pixels of the whole root fragment) x 100]. For this analysis, triplicates were used for root tissues grown in soil while duplicates were used for those grown in hydroponic culture for each time-point.

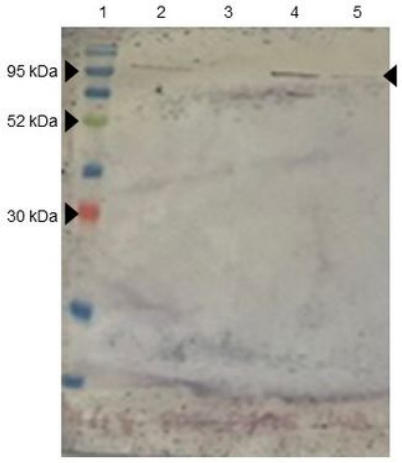

Figure 7

Western blot result showing the detection of 98kDa protein band in 9 mg/mL purified protein when 15 mg transformed A. tumefaciens crude protein were spiked to 15 mg total banana roots protein. Protein purification was done using nickel His GravitrapTM affinity column. m16D10 protein was detected in purification eluate (Lane 5) showing the band of ~98 kDa (shown by the arrow). Western blot assay was done using 2 µg anti-GUS primary antibody and 2 µg anti-mouse IgG- alkaline phosphatase, produced in goat secondary antibody. Lane 1: BLUltra Prestained Protein Ladder (GeneDireX, Taiwan); Lane 2: Crude protein sample of Agrobacterium tumefaciens cells harbouring pCAMBIA1304::m16D10::mgfp5::GUS::6xHis (positive control); Lane 3: Crude protein sample of untransformed A. tumefaciens cells (negative control); Lane 4: Crude protein mixture before purification.

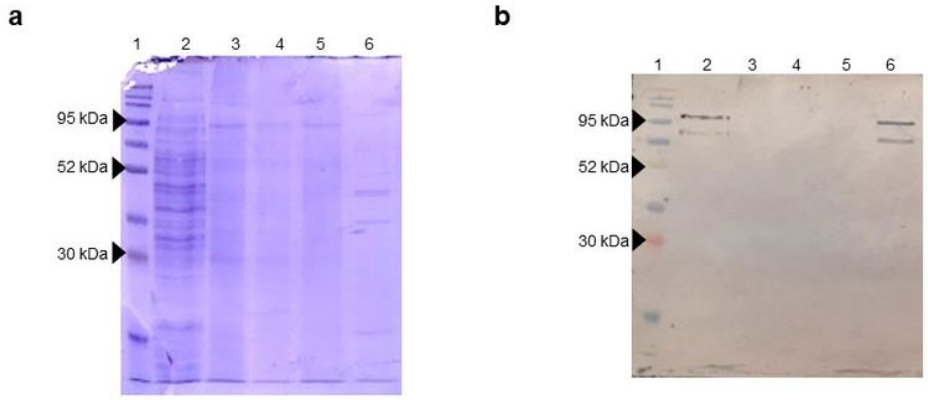

Figure 8

Protein purification results of putative banana root proteins interacting with m16D10 from transformed root tissues. Successful isolation of putative banana root proteins interacting with m16D10 observed in SDS-PAGE gel (a) , and the presence of transiently expressed m16D10 protein was confirmed with western blot assay using 2 µg anti-GUS primary antibody and 2 µg Anti-Mouse IgG- alkaline phosphatase, produced in goat secondary antibody (b). Analyses were conducted on transformed root tissues harbouring pCAMBIA1304::m16D10::mgfp5::GUS::6xHis grown in hydroponic media, harvested at 3-day post inoculation (dpi). Both SDS-PAGE and western blot assay results showed the presence of ~98kDa protein band indicating the presence of m16D10 protein (Lane 6). The presence of a smaller sized band may perhaps indicate the unspecific binding of antibodies used. Similar bands were detected in the positive control sample containing crude protein of Agrobacterium tumefaciens cells harbouring pCAMBIA1304::m16D10::mgfp5::GUS::6xHis (Lane 2).The presence of other protein bands of different sizes in Lane 6 of SDS-PAGE gel indicate the presence of putative banana root proteins interacting with m16D10 protein pulled-out during purification process using nickel His GravitrapTM affinity column. Lane 1: BLUltra Prestained Protein Ladder (GeneDireX, Taiwan); Lane 2: Crude protein sample of Agrobacterium tumefaciens cells harbouring pCAMBIA1304::m16D10::mgfp5::GUS::6xHis (positive control); Lane 3: Crude protein sample of untransformed A. tumefaciens cells (negative control); Lane 4: Flow-through product of nickel His GravitrapTM affinity column; Lane 5: Wash buffer product of nickel His GravitrapTM affinity column; Lane 6: Elution product of nickel His GravitrapTM affinity column.

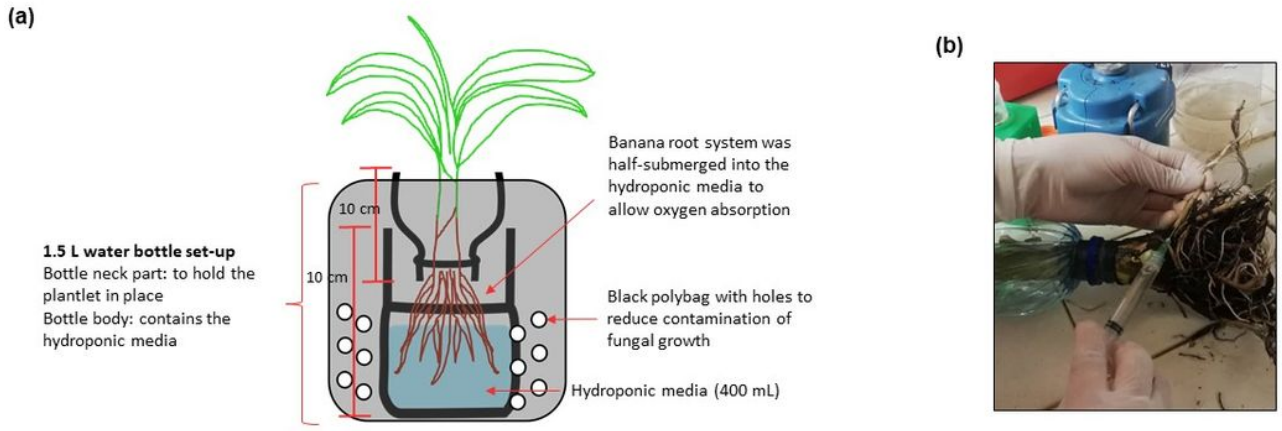

Figure 9

Transformation method of banana root tissues grown in an in-house hydroponic system. Transformation of banana root tissues cultured in (a) an in-house-designed hydroponic system used to acclimatise and grow banana plantlets for Agrobacterium-mediated transformation procedure in this study. The assemble was made of a 1.5 L used mineral water bottle. The upper part of the bottle (shoulder to neck part) was cut into 10 cm length, inverted, and used as a container to place the banana plantlet. The remaining part of the bottle was cut into 10 cm length and served as a base of the assemble where 400 mL of hydroponic media were placed. The assemble was then covered with a holed-black polybag to reduce fungal or moss growth and (b) syringe-based agroinfiltration technique conducted on the root tissues for in planta expression of pCAMBIA1304::CaMV35S::m16D10::mgfp5::GUS::6xHis.

Supplementary Files

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- SupplementaryTables.docx