Expression of Recombinant *Alcohol Dehydrogenase* in *Escherichia coli* Strain BL21 (DE3) and *In Planta Agrobacterium* Transformation of Tomato Seeds

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Abstract

Alcohol dehydrogenase is an enzyme that is involved in various roles in plant such as plant development, growth and plant responses to abiotic and biotic stresses. A recombinant *alcohol dehydrogenase 1* (*Adh1*) cDNA (r-*msAdh1*) from *Metroxylon sagu* has been previously isolated, and it contained 20 nucleotides derived from *Elaeis guineensis* at the 5'-end and had a molecular weight of 1.14 kb. The objective of this study is to determine the function of r-ms*Adh1* via analyses in prokaryotic and eukaryotic hosts. For expression in prokaryotic system, pET-41a(+) with a 8x His tag at the C terminal was used for r-msAdh1 protein purification, and expression was achieved using IPTG for 4-6 h in *Escherichia coli* strain BL21 (DE3) incubated at low temperature. The induced BL21 strain produced a small amount of soluble r-msAdh1 protein while a large amount was present as insoluble aggregates. Subsequently, the r-*msAdh1* cDNA was transformed into tomato seeds (*Solanum lycopersicum* cv. MT1) via *Agrobacterium*-mediated *in planta* transformation. The integration of r-*msAdh1* cDNA and the selectable marker were detected in transformed seedlings, T_0, using polymerase chain reaction technique. The transformation efficiency was determined to be 33% for r-*msAdh1* cDNA and 46% for the selectable marker. For stability analysis of the transgene, eleven T_1 generation randomly selected seedlings from the transgenic T_0 were analyzed for the presence of the cDNA, and all seedlings were found to contain the full length of r-*msAdh1* cDNA. However, out of the eleven T_1 transgenic lines produced, only four seedlings were used for expression analysis using the reverse transcriptase PCR (RT-PCR). Two transgenic lines, $T_1$9 and $T_1$11, were determined to contain r-*msAdh1* cDNA and this was verified by nucleotide sequencing. Although only a small number of T_1 transgenic seedlings was obtained, this study shows that tomato seeds could be used as a target tissue for *Agrobacterium*-mediated *in planta* transformation primarily because the protocol is easy, rapid and cheaper compared to tissue culture-based methods.

Keywords: *Alcohol dehydrogenase*, *Metroxylon sagu*, BL21 (DE3), *Agrobacterium tumefaciens*, tomato seeds
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1. Introduction

Alcohol dehydrogenase (ADH) is an enzyme that is actively expressed when plants are exposed to environmental stresses, e.g., anoxic or hypoxic conditions, osmotic stress, wound, dehydration and low-oxygen stress in water-logged roots [1- 4]. Apart from this, ADH is also involved in all stages of plant growth [5]. To better understand the role of ADH, expression studies have been conducted to produce soluble, bioactive ADH in various expression hosts [6-10]. While heterologous expression has been reported in many microorganisms, most of the research work has utilized the *E. coli* system because of the ease of genetic manipulation, and because of the availability of optimized expression vectors and host strains for the expression of eukaryotic proteins [11-13]. However, high levels of protein expression in *E. coli* often cause incorrect protein folding due to the bacteria's inability to carry out post-translational modifications, which results in aggregation of insoluble inclusion bodies (IBs) [14, 15]. For that reason, some *E. coli* strains, such as Rosetta (DE3), Rosetta-gami 2 (DE3) and *E. coli* strain BL21 (DE3), have been designed to enhance the expression of eukaryotic protein, and importantly these strains have been shown to successfully express soluble eukaryotic ADH [8-11]. To overcome the insolubility of proteins, induction at low temperature has been shown to alleviate the problem [9-10]. It works because the hydrophobic interactions that causes IBs to form are temperature dependent [16].

The expression in the eukaryotic host system provides several advantages such as easy genetic manipulation, high expression level, glycosylation ability and other eukaryotic post-translational modifications [17] compared to prokaryotic systems. Tomato (*Solanum lycopersicum* cv. MT1) is a member of *Solanaceace* family and abundant in genetic and genomic resources compared to other cultivated species [18]. It has also been used for development of new tools and plant models for genetic and genomic analyses [19]. Tomatoes have been used to study various aspects including herbicide tolerance [20], disease and insect resistance [21, 22], improvement in fruit quality [23], control in fruit ripening [24], improving yield [25], production of foreign protein [26] and food security [27]. Meanwhile, the *in planta Agrobacterium*-mediated transformation of tomato has been used to target various tissue sources such as mature and immature fruits [28, 29], shoot apical meristems [30], floral buds [28, 31] and flowers [28, 29]. However, the study of *in planta* transformation using tomato seeds has not been reported.

The activity of ADH enzyme in sago palm tissues has been previously identified with the highest expression detected in young leaves [1]. A full length r-*msAdh1* cDNA (MN196247) has also been isolated using the rapid amplification cDNA ends (RACE) technique [32]. Here, we report on the expression analysis of r-*msAdh1* cDNA in bacteria and the transformation into tomato plants via *in planta Agrobacterium*-mediated transformation of tomato seeds. The r-*msAdh1* cDNA was firstly cloned into a bacterial expression vector, pET-41a(+), fused to a histidine tag and was expressed in *E. coli* BL21 (DE3). The r-*msAdh1* cDNA was then cloned into a binary vector, pGSA1131, and transformed into tomato seeds.

2. Materials and Methods

2.1 Cloning of recombinant *Adh1* (r-*msAdh1*) *cDNA* into pET41a(+) and expression in *E. coli* strain BL21 (DE3)

2.1.1 Directional cloning of r-*msAdh1* cDNA into pET-41a(+)

The open reading frame of r-*msAdh1* was obtained through PCR by using the following primers: 5'NdeI_adaptor (5'-GGAATTCCATATGGCAAGCACTGTTGG-3') and 3_r-msAdh1_XhoI (5'-

GCTAACTCGAGACCATCCATGTGAATGATGCAC-3'). A 1X PCR mixture was prepared to include 2.5 µl 10x High Fidelity buffer with 15 mM $MgCl_2$, 2.5 µl dNTP (2.5 mM), 0.5 µl high fidelity DNA polymerase enzyme (0.625 U/µl) (Thermo Fisher Scientific, USA), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 17 µl nuclease-free water and 0.5 µl template (10 pmol). The template used in the PCR was pET-41a(+) that contained r-*msAdh1* cDNA with seven stop codons before the His-tag sequence. The PCR was conducted according to the program that includes hotstart at 94°C for 3 min; 35 cycles of 94°C for 30 s, 62°C for 1 min, 72°C for 2 min and a final extension of 72°C for 10 min. The amplified r-*msAdh1* fragment and pET-41a(+) (Novagen, USA) were then subjected to double restriction enzyme digests of *Nde*I and *Xho*I (Thermo Fisher Scientific, USA). The restricted r-*msAdh1* and pET-41a(+) fragments were then ligated using T4 Ligase (Thermo Fisher Scientific, USA) to produce the construct pET-41a(+)/r-*msAdh1*, and transformed into *E. coli* strain XL-1 Blue via heat shock method. Positive colonies were screened on the Luria agar (LA) supplemented with 100 µg/ml kanamycin. The XL-1 blue culture harboring the recombinant plasmid was extracted and the nucleotide sequence was verified via sequencing (Apical Scientific, Malaysia) and restriction enzyme analysis.

2.1.2 Induction and expression of pET-41a(+)/r-*msAdh1* in BL21 (DE3)

For expression in *E. coli*, the pET-41a(+)/r-*msAdh1* was transformed into BL21 (DE3) competent cells via heat shocked method. A single colony of BL21 (DE3) harboring pET-41a(+)/r-*msAdh1* was selected and used to inoculate 6 ml Luria broth (LB) supplemented with 100 µg/ml kanamycin. The culture was grown to OD_{600} of 0.6-1.0 at 37°C and kept at 4°C for overnight. Two milliliters of the overnight culture was pipetted out and centrifuged for 30 s at 3500 rpm. The supernatant was discarded and pellet was re-suspended with 1 ml fresh LB media. The suspension was added with 49 ml LB media supplemented with 100 µg/ml kanamycin and transferred into 250 ml Erlenmeyer flask. The culture was brought to OD_{600} of 0.5-0.6 at 37°C. Once the culture reached the desired OD, 1.5 ml culture was aliquoted to serve as non-induced control. Ten milliliters of the culture was then pipetted into flasks; A, B, C and D, and induced with IPTG with a final concentration of 0.4 mM. Flasks A and B were incubated for 4 h (T4) and 6 h (T6) respectively, at 15°C. Meanwhile, flasks C and D were incubated for 4 h (T4) and 6 h (T6) respectively, at 27°C. At the end of incubation, OD_{600} of all cultures was measured. Next, 1.5 ml of each culture was aliquoted and centrifuged at 13,200 rpm for 4 min. The pellet was stored at -20°C until use. The procedure for bacterial expression was carried out as recommended by the QIAexpressionist™ Kit (Qiagen, Germany) with minor modifications.

2.1.3 Determination of protein solubility, purification under native condition and enzymatic assay of ADH

To determine the solubility of r-msAdh1 protein, the cell pellet was lysed using NP10 lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole). The volume of lysis buffer used was in accordance with cell culture density (OD_{600} = 0.1, 12.5 µl of NP10 lysis buffer, OD_{600} = 0.6, 75 µl of NP10 lysis), and the total protein concentration was standardized. The crude lysate was centrifuged, soluble and insoluble fractions were analyzed on 12% SDS polyacrylamide gel electrophoresis (PAGE) (Bio-Rad, USA). Meanwhile, for protein purification, 20 ml culture of BL21 (DE3) with r-*msAdh1* expression was used. The purification was done by using Ni-NTA Spin Kit (Qiagen, Germany). The result was analyzed on 12% SDS-PAGE. The catalyzing activity of r-msAdh1 was analyzed spectrophotometrically at OD_{340} [33]. The increase in absorbance was recorded at every one min for the first 15 min as NAD^+ was reduced to NADH. The reaction buffer consisted of 1 ml Tris-HCl 100 mM pH 8.3, 80 µl ethanol and 2 mM NAD^+ and incubated at 25°C. The reaction was initiated by adding 20 µl of r-msAdh1 lysate [33].

2.2 Cloning of r-*msAdh1* cDNA into pGSA1131 and *in planta Agrobacterium* transformation of tomato seeds

2.2.1 Directional cloning of r-*msAdh1* cDNA into pGSA1131

The open reading frame of r-*msAdh1* cDNA was obtained through PCR amplification by using the following primers: 5_NcoI_msAdh1 (5'-GGAATTCCATGGCAAGCAGTGTTGGTCAA-3') and 3_BamHI_msAdh1 (5'-ACCAAGGATCCTTAGTGGTGGTGGTG-3'). A 1X PCR mixture was prepared to include 2.5 µl 10x High Fidelity buffer with 15 mM $MgCl_2$, 2.5 µl dNTP (2.5 mM), 1 µl high fidelity DNA Polymerase enzyme (0.625 U/µl) (Thermo Fisher Scientific, USA), 1 µl forward primer (10 µM), 1 µl reversed primer (10 µM), 0.5 µl template (pET-41a(+)_r-*msAdh1*) (10 pmol) and 16 µl nuclease-free water. The PCR was conducted according to the program that includes hotstart at 94°C for 3 min; 35 cycles of 94°C for 30 s, 64°C for 1 min, 72°C for 1 min and 30 s and 1 cycle of 72°C for 10 min. The amplified r-*msAdh1* fragment and pGSA1131 were then subjected to double restriction enzyme digests of *Bam*HI and *Nco*I (Thermo Fisher Scientific, USA). The restricted pGSA1131 fragment (approximately 9 kb) was recovered from the agarose gel using Gel DNA Extraction Kit (Vivantis, Malaysia) and subsequently ligated with r-*msAdh1* using T4 DNA Ligase (Thermo Fisher Scientific, USA). The ligated mixture was then transformed into *E. coli* strain XL1 Blue via heat shock method. Putative transformants were selected on the Luria agar (LA) supplemented with 30 µg/ml chloramphenicol. Clones harbouring pGSA1131/r-*msAdh1* were isolated and analyzed via nucleotides sequencing (Apical Scientific, Malaysia) and restriction enzyme digestion analysis.

2.2.2 Preparation of *A. tumefaciens* culture, infiltration and post-infiltration broth

Agrobacterium tumefaciens strain LBA4404 was kindly provided by Evra Raunie Ibrahim from Craun Research Sdn. Bhd. (Malaysia). The transformation of pGSA1131/r-*msAdh1* into *A. tumefaciens* using Bio-Rad Gene Pulser Xcell Electroporation Systems (Bio-Rad, USA) was according to method described by manufacturer. Positive colony obtained from electroporation was cultured in LB for 2 days. Six hundred microliters was then added into a 30 ml fresh LB supplemented with 100 µg/ml rifampicin and 30 µg/ml chloramphenicol. The culture was brought to OD_{600} of 0.5-0.6 and centrifuged at 3500 rpm for 5 min. The cell pellet was then re-suspended in an infiltration media (0.5X MS; 3% sucrose; 0.5 g/l MES). Prior to co-cultivation with tomato seeds, the infiltration media was added with Silwet L-77 (0.003%, PhytoTechnology Laboratories, USA) and 200 µM acetosyringone (PhytoTechnology Laboratories, USA).

The tomato (*Solanum lycopersicum*) cultivar MT1 was purchased from Malaysia Research Institute, MARDI (Malaysia). Fifty seeds were incubated in sterilized distilled water for overnight at 4°C. The following day, the seeds were sonicated for 10 min prior to addition of the infiltration media containing Silwet L-77 and acetosyringone. The seed co-cultivation was agitated for 3 h at 180 rpm. After 3 h, the infiltration media was removed and the seeds were rinsed with distilled water several times. The seeds were then treated with 500 µg/ml carbenicillin for an hour to remove any remaining *A. tumefaciens*. The seeds were sown on soil and germinated after 3-5 days.

2.2.3 Analysis of r-*msAdh1* integration in T_0 and T_1 generations using PCR

To analyze the r-*msAdh1* integration in T_0 and T_1 generations, young leaf of putative transgenic seedlings of approximately 0.5 cm^2 was used for genomic DNA extraction. The leaves were surface-sterilized for 2 min in 75% ethanol and 10% Clorox® bleach, and then followed by three

washes using sterilized distilled water. Genomic DNA was extracted using GF-1 Plant DNA Extraction Kit (Vivantis, Malaysia) according to instructions provided by manufacturer. The quantity and purity of the extracted genomic DNA (gDNA) was measured using Ultrospec 1100 Pro UV/Vis Spectrometer (Amersham Pharmacia Biotech, USA). The integration of r-*msAdh1* in putative transformed T_0 seedlings (1-15), and transgenic T_1 seedlings (1-11), were screened by PCR using r-*msAdh1* specific primers: 5_Comseq_Adh (5'-ATGGCAAGCAGTGTTGGTCAAGTGATC-3') and 3_Comseq_Adh (5'- ACCATCCATGTGAATGATGCACCTAAGGC-3'). A 1X PCR mixture was prepared to include 7.5 µl of 2X Green GoTaq master mix (Promega, USA), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 1 µl genomic DNA (0.5 µg/µl) and 4.5 µl nuclease-free water. The PCR was conducted according to the program that includes hotstart at 94°C for 3 min; 35 cycles of 94°C for 30 s, 57°C for 45 s, 72°C for 1 min and 30 s and 1 cycle of 72°C for 10 min.

2.2.4 Analysis of *bar* gene integration in T_0 generation using PCR

The integration of *bar* gene in putative transformed seedlings (T_0 generation) was analyzed using gradient PCR at annealing temperature ranging from 55°C-68°C. The *bar* gene specific primers were used, namely the Bar3_F (5'-ATGAGCCCAGAACGACGCC-3') and Bar3_R (5'-ATCTCGGTGACGGGCAGG-3'). The composition of 1X PCR mixture to detect the presence of *bar* gene was 2.5 µl 10x High Fidelity buffer with 15mM $MgCl_2$, 2.5 µl dNTP (2.5 mM), 1 µl high fidelity DNA Polymerase enzyme (0.625 U/µl) (Thermo Fisher Scientific, USA), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 1 µl genomic DNA (0.5 µg/µl), and 16 µl nuclease free water. The PCR was conducted according to the program that includes hotstart at 94°C for 3 min; 35 cycles of 94°C for 30 s, 55-68°C for 45 s, 72°C for 30 s and 1 cycle of 72°C for 10 min.

2.2.5 Analysis of r-*msAdh1* expression by using reverse-transcription PCR

To analyze r-*msAdh1* expression in tomato putative transformants, only $T_0$2 and $T_1$8, $T_1$9, $T_1$11 lines survived and used in subsequent analysis. Transformed seedling at the 4-5 leaves stage was used for analysis. Young leaf with size approximately of $1cm^2$ was surfaced sterilized and ground to a fine powder with liquid nitrogen in a pre-cooled mortar. Total RNA extraction was performed using Total RNA Mini Kit Plant (Geneaid, Taiwan) according to instructions provided by manufacturer and treated with *DNAse1* (Promega, USA) via standard protocol. The quantity and purity of the total RNA obtained was measured using Ultrospec 1100 Pro UV/Vis Spectrometer (Amersham Pharmacia Biotech, USA). Meanwhile, total RNA integrity was accessed on 1.5% of agarose gel. First strand cDNA synthesis of r-*msAdh1* was carried using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to manufacturer recommendation. Three primers were used for the first strand DNA synthesis: 3_Comseq_Adh (5'-ACCATCCATGTGAATGATGCACCTAAGGC-3'), 3_BamHI_msAdh1 (5'-ACCAAGGATCCTTAGTGGTGGTGGTG-3') and 5_msAdh1_R (5'-AACACAGCCAAC ATGGACAA-3') (Figure 1). The presence of r-*msAdh1* expression was determined by RT-PCR using combination of r-*msAdh1* cDNA specific forward and reverse primers: 5_Comseq_Adh (5'-ATGGCAAGCAGTGTTGGTCAAGTGATC-3') and 5'_msAdh1_R (5'-AACACAGCCAAC ATGGACAA-3'). The reaction mixture for amplification includes 12.5 µl 2X Dream-Taq Green PCR Master Mix (Promega, USA), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 2 µl cDNA, 9.5 µl nuclease-free water. The PCR was conducted according to the program that includes hotstart at 94°C for 3 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 45 s and 1 cycle of 72°C for 5 min.

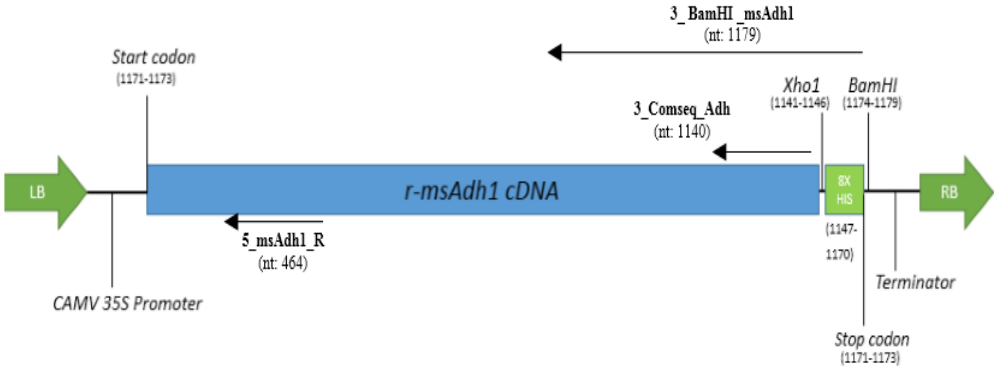


Figure 1. The orientation and position of primers used for first strand cDNA synthesis reaction

3. Results and Discussion

3.1 Determination of protein solubility, purification under native condition and enzymatic assay of ADH

The use of primers combination; 3'XhoI_r-msAdh1 and 5'NdeI_adaptor in PCR to amplify r-*msAdh1* cDNA resulted in amplification of approximately 1.1 kb fragment (Figure 2). Sequence analysis showed that r-*msAdh1* cDNA has been successfully cloned into pET-41a(+) with length of 1.14 kb corresponding to 380 amino acids with C-terminal His-tag fusion. Meanwhile Figure 3 is a PAGE analysis that shows presence of small amount of soluble r-msAdh1 (Lane 2-5, 42.55kD) and a large amount of r-msAdh1 expressed as insoluble fraction (Lanes 6-9, 42.55 kD). The intensity of the bands increased after induction temperature was increased to 27°C for a period of 6 h. Over-expression of heterologous protein causes *in vivo* equilibrium to favor the formation of inclusion bodies (IBs) rather than solubilized protein due to the translation rate exceeding the rate of protein folding [34, 35]. The formation of IBs is hard to predict even with *in silico* analysis of amino acid sequence [36] because they are complex and the aggregates are only formed within similar type of protein or highly similar protein [35, 15]. Analysis using the software GenScript Rare Codon Analysis (https://www.genscript.com/tools/rare-codon-analysis) showed that r-*msAdh1* cDNA has codon adaptation index (CAI) of 0.62 which is below the ideal value (0.8-1.0) to obtain good expression. The strain BL21 (DE3) used in this study was not designed to enhance the expression of eukaryotic gene that has rarely used codons in *E. coli.* In contrast, a large amount of soluble tag protein (GST, 8xHis and S-tag; nucleotides: 1095-150) with molecular weight of 33.71 kD was expressed as seen in Lanes 10-13.

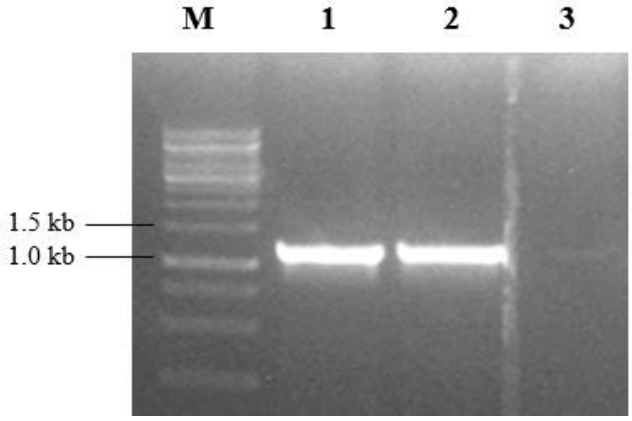


Figure 2. Gel electrophoresis of PCR product visualized on 1% agarose gel stained with EtBr. The PCR product is the full length of r-*msAdh1* cDNA fragment that amplified using the 5'NdeI_adaptor and 3'XhoI_r-msAdh1 primers combination. M: GeneRuler™ 1 kb DNA Ladder (Thermo Fisher Scientific, USA), 1 and 2: PCR products, 3: Negative control (PCR without template)

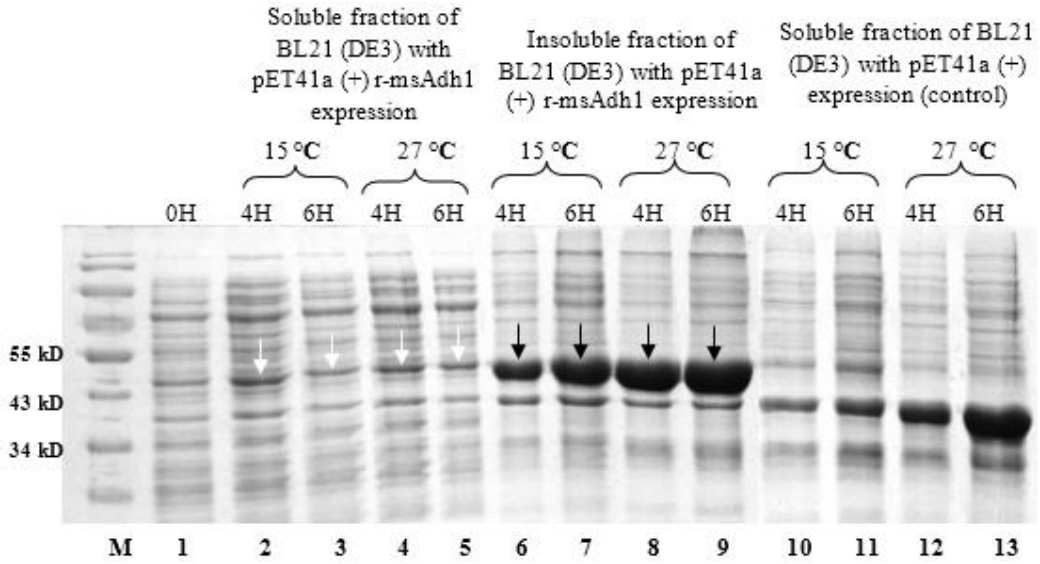


Figure 3. Crude protein analyzed on 12% SDS-PAGE stained with Coomassie Brilliant Blue 1: Soluble fraction of non-induced culture of BL21 (DE3) with r-*msAdh1* expression (0 h). 2-5: Soluble fraction of BL21 (DE3) with r-*msAdh1* expression at 15°C and 27°C for 4 h and 6 h, respectively. 6-9: Insoluble fraction of BL21 (DE3) with r-*msAdh1* expression at 15°C and 27°C for 4 h and 6 h, respectively. 10-13: Soluble fraction from crude lysate of BL21 (DE3) with pET-41a(+) expression (control). M: EZ Run pre-stained *Rec* protein ladder. White arrows show the expected soluble r-msAdh1 protein bands and black arrows show the expected insoluble r-msAdh1 protein bands

Purification of r-msAdh1 protein was successful, however several non-target protein was also co-eluted (Figure 4). The formation of disulphide bond between cellular host protein and protein in interest could lead to contamination during purification [37]. Formation of inclusion bodies and/or targeted protein tertiary structure, would also block the efficacy of polyhistidine affinity tag [37]. ADH enzyme breaks down ethanol to form acetaldehyde in the presence of NAD^+ as co-enzyme. The catalytic activity of r-msAdh1 can be monitored by the increase of absorbance reading. This is because reduced NAD^+ (NADH) exhibits strong UV absorption at 340 nm while the NAD^+ has almost no absorption at this wavelength. Figure 5 shows catalytic activity of ADH enzyme was detected not only in the soluble fraction of BL21 (DE3) with expressed r-*msAdh1* but also in the control. This is expected because endogenous ADH is known to be present in BL21 (DE3) [38]. Nevertheless, Independent t-test showed a p-value of $0.003 < 0.05$ indicating a significant difference between the catalytic activity of ADH enzyme present in soluble fraction of BL21 (DE3) with expressed r-*msAdh1* and control BL21 (DE3) containing only pET-41a(+).

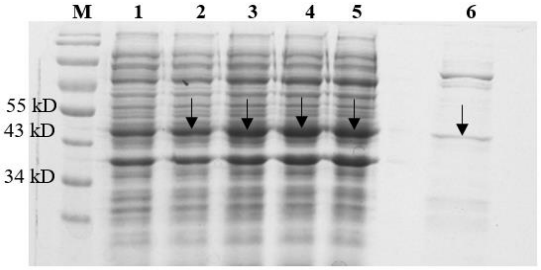


Figure 4. Crude lysate of BL21 (DE3) and Ni-NTA purified r-msAdh1 analyzed on 12% SDS-PAGE stained with Coomassie Brilliant Blue. 1: Soluble fraction of non-induced culture of BL21 (DE3) with r-*msAdh1* expression (0 h). 2-5: Soluble fraction of BL21 (DE3) with r-*msAdh1* expression at 15°C and 27°C for 4 h and 6 h, respectively. 6: Purification of r-msAdh1 protein using 50 mM NaH_2PO_4, 300 mM NaCl and 500 mM imidazole as elution buffer. M: EZ Run pre-stained *Rec* protein ladder. Expected r-msAdh1 bands are shown by black arrows.

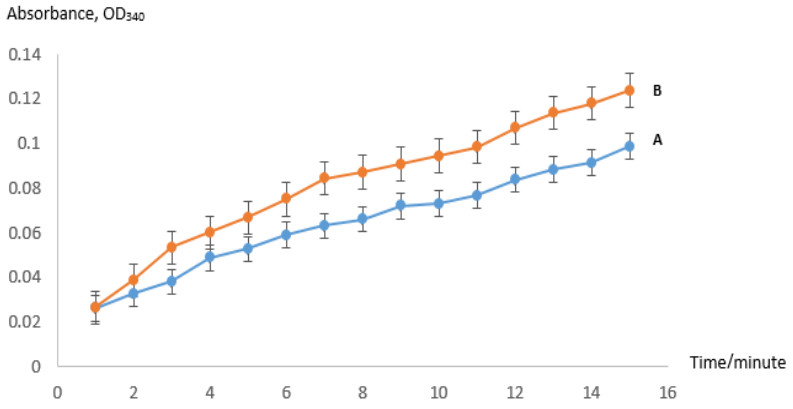


Figure 5. Catalytic activity of ADH enzyme. Absorbance at OD_{340} versus time (min) obtained from (A) soluble fraction of BL21 (DE3) transformed with pET-41a(+) (control) and, (B) soluble fraction of BL21 (DE3) with expressed r-*msAdh1*. Graph plotted with standard deviation (SD) error bar and each point represents the average of triplicate.

3.2 Analysis of r-*msAdh1* and *bar* integration in tomato T_0 and T_1 generations

The use of primers combination; 5_NcoI_msAdh1 and 3_BamHI_msAdh1 in PCR to amplify r-*msAdh1* cDNA resulted in amplification of 1.14 kb fragment on 1% agarose gel electrophoresis (Figure 6). For tomato transformation, the r-*msAdh1* cDNA was cloned after the CaMV35S promoter of the binary vector, pGSA1131. This was confirmed via restriction enzyme analysis and nucleotide sequencing. After seed transformation, a total of 50 seeds germinated but only fifteen putative seedlings (T_0) survived and were used to determine r-*msAdh1* integration. Two negative controls were used in PCR; genomic DNA extracted from wild type tomato and PCR mix without any template, meanwhile a pGSA1131_r-*msAdh1* recombinant plasmid was used as positive control. PCR analysis and sequencing of the PCR fragments of the T_0 seedlings DNA showed five putative transformed plant lines ($T_0$1, $T_0$2, $T_0$4, $T_0$12, $T_0$13) contained the r-*msAdh1* cDNA (Figure 7). Analysis of T_1 generation obtained from $T_0$2, $T_0$12 and $T_0$13 showed that $T_1$1- $T_1$11, also contained r-*msAdh1* (Figure 8). However, r-*msAdh1* in $T_1$4 and $T_1$11 were only detected at annealing temperatures of 60.1°C, 61.1°C and 61.7°C (Figure 9) and using RT-PCR (Figure 12), respectively.

There was no amplification of *bar* gene detected from all tested plants when dimethyl sulfoxide (DMSO) or bovine serum albumin (BSA) was added in PCR reaction. This is possibly due to the high GC content (68%) and the presence of continuous block of GC nucleotides [39] that interferes the annealing of primer to the DNA template [40]. The amplification of *bar* gene was only obtained in gradient PCR by using high fidelity DNA Polymerase enzyme (Thermo Fisher Scientific, USA) at annealing temperatures of 62.4°C, 64.0°C and 65.6°C (Figure 10). Higher annealing is necessary for primer with high GC content [41]. Out of fifteen plants tested, seven putative transformed plant lines: $T_0$1, $T_0$3, $T_0$4, $T_0$5, $T_0$9, $T_0$12 and $T_0$13 were positive for *bar* gene (Figure 11); thus resulting in higher transformation efficiency (46.7%) compared to r-*msAdh1* (33.33%). Perhaps, the *bar* gene is smaller and easier to incorporate into plant genome [42]. Besides, from the result obtained, it showed that both of r-*msAdh1* and *bar* gene did not always co-integrate in transformed plants. The result was in line with other study that found that the copy number of *bar* and *gus* genes on the same T-DNA was different in individual transformants [43] which could be due to rearrangements or truncated T-DNA [44] caused by induction of CaMV35S promoter [45]. The selection of transgenic plant with glufosinate could not be performed as most of transformed plants showed fatality when they reached stage of 4-6 leaves that could be due to overexpression of r-*msAdh1* or excessive cutting during sampling for DNA and RNA extraction.

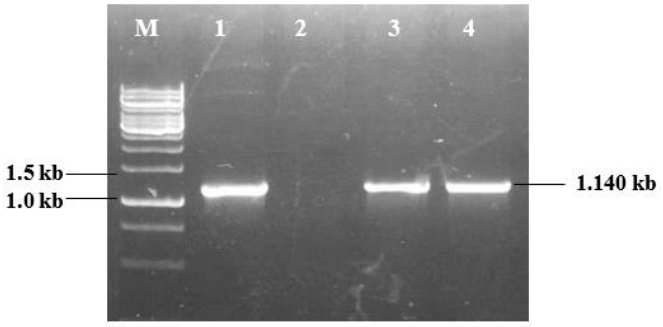
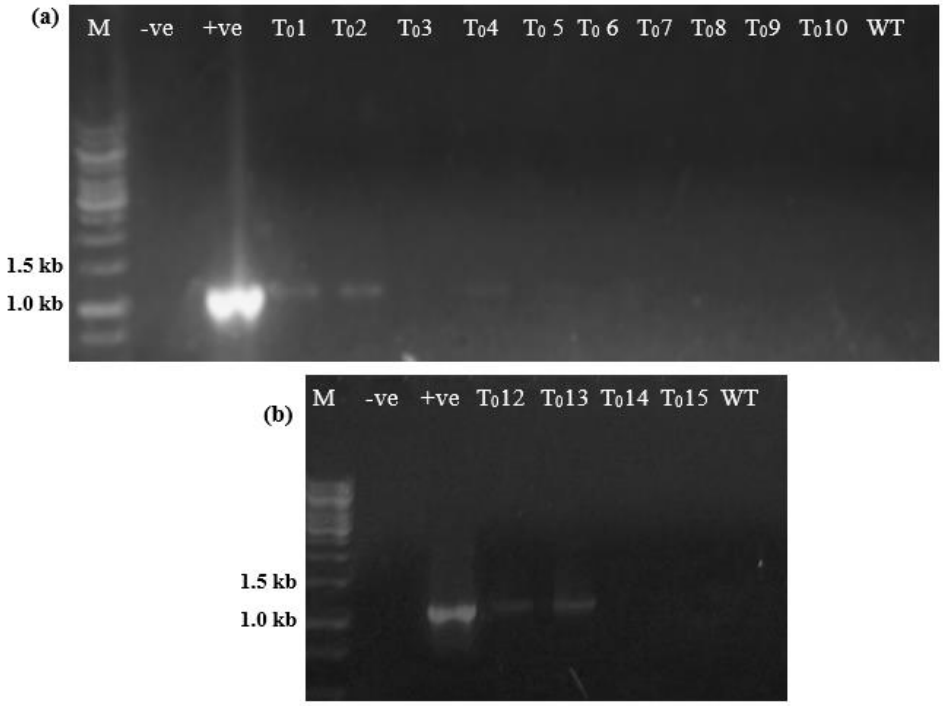


Figure 6. Gel electrophoresis of r-*msAdh1* amplification visualized on 1% of agarose gel stained with EtBr. The used of 5'NcoI_msAdh1 and 3_BamHI_msAdh1 primers in PCR produced r-*msAdh1* band with expected size of 1.14 kb. M: GeneRuler™ 1 kb DNA Ladder (Thermo Fisher Scientific, USA), 2: Negative control, 1, 3 and 4: PCR product



Figures 7. Diagnostic analysis of T_0 generation of tomato seedlings. Gel electrophoresis of PCR product visualized on 1% agarose gel stained with EtBr to detect r-*msAdh1* cDNA in the putative transformed seedling, T_0. The PCR product is the full length of r-*msAdh1* cDNA fragment amplified using 5_Comseq_Adh and 3_Comseq_Adh primers combination. M: GeneRuler™ 1 kb DNA Ladder (Thermo Fisher Scientific, USA), –ve: Negative control (PCR without DNA template), +ve: Positive control, $T_0$1- $T_0$15: Putative transformed seedling 1-15, WT: Wild type control

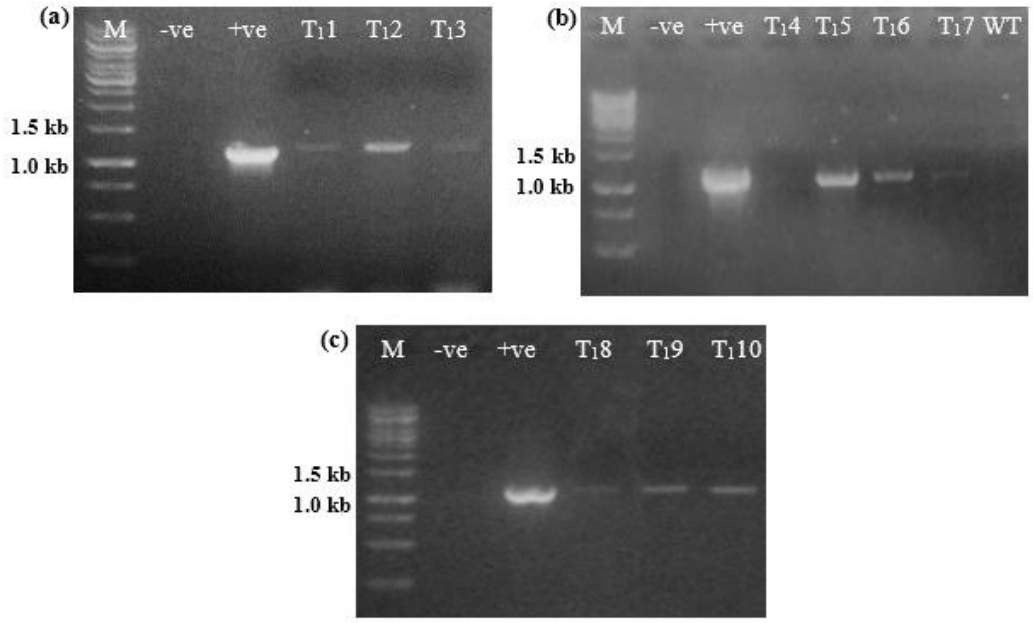


Figure 8. Diagnostic analysis of T_1 generation of tomato seedlings. Gel electrophoresis of PCR product visualized on 1% agarose gel stained with EtBr to detect r-*msAdh1* in T_1 generation from $T_0$12 (a), $T_0$13 (b) and $T_0$2 (c). The PCR product is the full length of r-*msAdh1* cDNA fragment amplified using 5_Comseq_Adh and 3_Comseq_Adh primers combination. M: GeneRuler™ 1 kb DNA Ladder (Thermo Fisher Scientific, USA), –ve: Negative control, +ve: Positive control, $T_1$1-$T_1$10: T_1 generation 1-10, WT: Wild type control

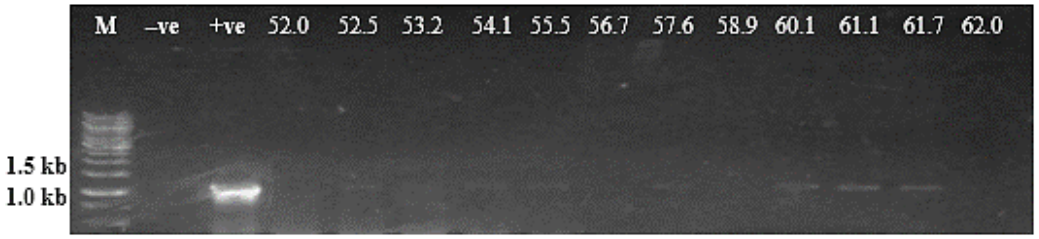


Figure 9. Diagnostic analysis of T_1 generation of tomato seedling. Gel electrophoresis of gradient PCR products on 1% agarose gel stained with EtBr to detect r-*msAdh1* in $T_1$4 generation from $T_0$13. The PCR product is the full length of r-*msAdh1* cDNA fragment amplified using 5_Comseq_Adh and 3_Comseq_Adh primers combination. M: GeneRuler™ 1 kb DNA Ladder (Thermo Fisher Scientific, USA), –ve: Negative control, +ve: Positive control. 52.0°C-62.0°C: Annealing temperatures

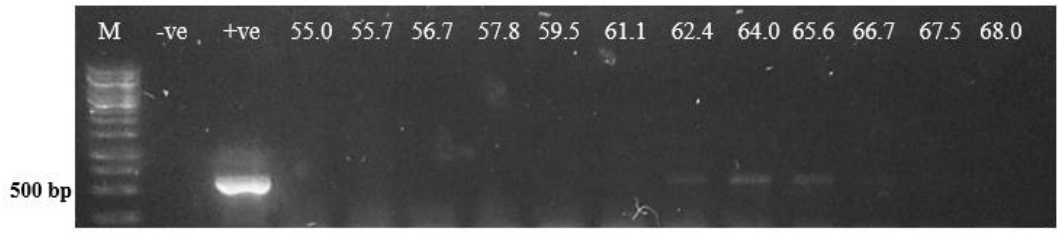
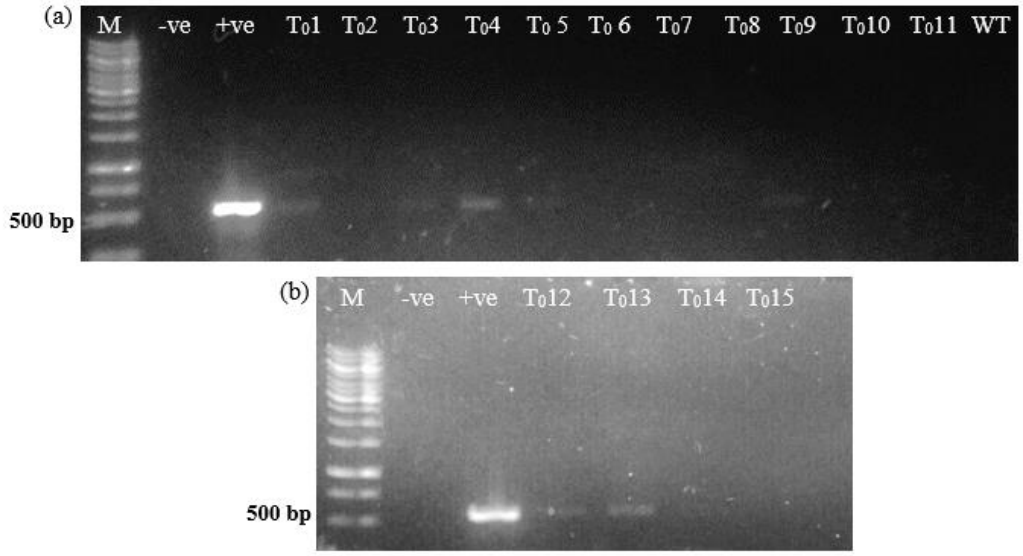


Figure 10. Gel electrophoresis of gradient PCR products for *bar* gene on 1.5% agarose gel stained with EtBr. Genomic DNA extracted from putative transformed seedling; $T_0$1 was used as a template in gradient PCR. M: GeneRuler™ 1 kb DNA Ladder (Thermo Fisher Scientific, USA), –ve: Negative control (PCR without DNA template), +ve: Positive control. 55.0°C-68.0°C: Annealing temperatures



Figures 11. Gel electrophoresis of *bar* gene PCR products from T_0 analyzed on 1.5% agarose gel stained with EtBr. The PCR product is the full length *bar* gene fragment amplified using Bar3_F and Bar3_R primers. M: GeneRuler™ 1 kb DNA Ladder (Thermo Fisher Scientific, USA), –ve: Negative control (PCR without DNA template), +ve: Positive control (PCR using pGSA1131/r-*msAdh1* plasmid as a template), $T_0$1- $T_0$15: Putative transformed seedling, 1-15. WT: Wild type (control).

Transformation efficiency using *A. tumefaciens* differs and is determined by the *Agrobacterium* strains, binary vectors, target tissues used and plant species. Previous works in *in planta* transformation such as in using the technique of *in vitro* tomato fruit injection method, reported transformation efficiency of between 17-68% for tomato infiltrated with *A. tumefaciens* strain EHA105 harboring the plasmid pROKII carrying *Apetala 1* (*Ap1*) and 21% for pROKII carrying *LEAFY* (*LFY*) gene [28, 46]. Meanwhile, tomato infiltrated with *A. tumefaciens* strain LBA4404 plasmid harboring plasmid pPM7 with *HAL1* and *NPTII* genes achieved transformation rate of between 34-42% [29]. All of these experiments used ripened tomato as a target tissue at 48 h incubation after infiltration. On the use of ripen and immature fruits as target tissues using the

in vivo fruit injection method, varying transformation efficiencies were also reported between 35-42% for ripened tomato and 2-5% for immature/green tomato [28]. On the other hand, the floral-dip method targets the ovules, where unopened flowers are introduced to *Agrobacterium* before pollination and subsequently left to develop into flower [28, 39]. Using the floral-dip method, transformation efficiency in range of 12-23% was obtained [28]. This work reports the first *in planta* transformation using tomato seeds as a target tissue and producing a transformation efficiency of between 33.3-46.7%.

3.3 Molecular analysis of r-*msAdh1* expression in transformed seedlings

Most of transformed seedling showed fatality when they reached the stage with 4-6 leaves. Therefore, total RNA was extracted only from four available positive lines; $T_1$8, $T_1$9, $T_1$10 and $T_1$11. RNA purity of between 1.8-2.0 indicates good RNA quality for $T_1$9 and $T_1$11 samples. A lower purity was obtained for samples $T_1$8 and $T_1$10 indicating RNA contamination with protein or phenol. The RT-PCR using r-*msAdh1* specific primers; 5_Comseq_Adh1 and 5_msAdh1_R and subsequent agarose gel electrophoresis produced the expected 500 bp fragment (Figure 11), indicating the presence of r-*msAdh1* transcript in both of the transgenic progeny seedlings of $T_1$9 and $T_1$11. However, no amplification was detected from samples $T_1$8 and $T_1$10, which may be due to the poor RNA quality (data not shown). However, the RT-PCR obtained only when the r-*msAdh1* specific internal specific primer, 5_msAdh1_R, was used to generate the first cDNA strand. This could be because the first cDNA strand was truncated when using the 3_Comseq_Adh1 or 3_BamH1_msAdh1 as primers (Figure 10) due to the presence of mRNA secondary structures that affects the activity of reverse transcriptase enzyme [47].

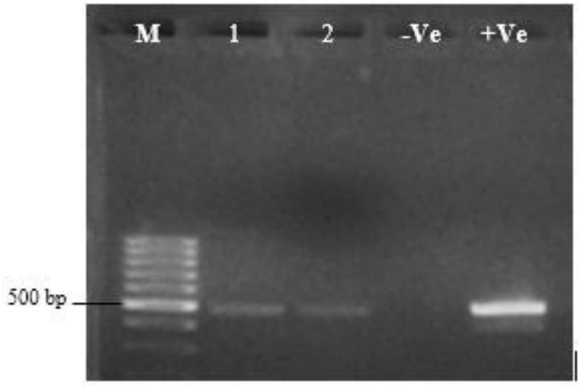


Figure 12. Gel electrophoresis of RT-PCR product visualized on 1.5% agarose gel stained with EtBr to analyze the expression of r-*msAdh1* cDNA in transformed seedlings. Lanes 1 and 2 shows a 500 bp size fragments representing the r-*msAdh1* cDNA amplified using 3_comseq_Adh and 5_msAdh1_R primers. M: GeneRuler™ 1 kb DNA Ladder (Thermo Fisher Scientific, USA), –ve: Negative control (PCR without DNA template), +ve: Positive control, 1: $T_1$9, 2: $T_1$11

4. Conclusions

The r-*msAdh1* cDNA in BL21 (DE3) was highly expressed in *E. coli* system. However, most of the protein formed as insoluble inclusion bodies, even when low temperatures (15°C and 27°C) and low concentration of IPTG were used. We have also demonstrated a successful transformation method by using *Agrobacterium*-mediated *in planta* seed transformation protocol for the transfer of *msAdh1* cDNA into the tomato genome. This method is an alternative for functional analysis of r-msAdh1 protein as the transformation is easy, rapid and economical, with transformation efficiency of between 33.3-46% within two weeks. This simple and easy to perform method can provide a platform for the further characterization of not only r-*msAdh1* but also other eukaryotic genes in plant systems.

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