ISSN: 1511-3701
Pertanika J. Trop. Agric. Sci. **35 (2)**: 371 - 379 (2012)

Over-expression of *Escherichia coli* Transaldolase in the Cytosol of *Arabidopsis thaliana*

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ABSTRACT

Transaldolase (TAL) is an enzyme of the oxidative pentose phosphate pathway (OPPP) which catalyzes the reversible reaction of sedoheptulose-7-phophate into fructose-6-phosphate and erythrose-4-phosphate. In some micro-organisms, fungi and plants, erythrose-4-phosphate condenses with phosphoenolpyruvate (PEP) from glycolysis to form chorismate which is a precursor for many secondary metabolic pathways such as aromatic amino acids, flavonoids, lignin, indole acetate and UV light protectants. An analysis of plant genome databases reveals that the OPPP is incomplete in the cytosol of plants as no genes encoding for a cytosolic transaldolase (TAL) and transketolase (TK) have been identified so far. Thus, this study attempted to complete the compartmentation of TAL in the cytosol and plastid of plants by over-expressing it in the cytosol of *A. thaliana*. For this purpose, homozygous transgenic plants were obtained in these studies; it was found that the transaldolase activity of transgenic lines increased as compared to wild type plants. The findings of the current study also demonstrated that transgenic plants did not show any distinct phenotypes and there was no difference in a range of growth parameters compared with *A. thaliana* Col-0 (wild type).

Keywords: ***Agrobacterium tumefaciens,*** **transaldolase, oxidative pentose phosphate pathway, transgenic plants, shikimate pathway**

INTRODUCTION

Oxidative Pentose Phosphate Pathway (OPPP) is involved in the metabolism of carbohydrates *via* the oxidation of glucose-6-phosphate. The pathway is composed of two phases, namely, the oxidative and the non-oxidative. The first phase is irreversible and it consists of the oxidation of glucose-6-phosphate that leads to the production of ribulose-5-phosphate. A large percentage (50-60%) of the required NADPH, a major reducing power for various anabolic pathways including the biosynthesis of fatty acids, the reduction of nitrite (Sprenger, 1995; Dennis *et al.*, 1997; Debnam *et al.*, 2004), for cell protection against oxidative stress and synthesis of glutamate (Schnarrenberger *et al.*, 1995) is contributed by the first phase. The second phase consists of a reversible series of interconversion between 3-, 4-, 5-, 6- and 7-carbons sugar that are catalyzed by the enzymes ribose-5-phosphate isomerase (RPI), ribulose-5-phosphate-3-epimerase (RPE) and transketolase (TK) and transaldolase (TAL).

In some micro-organisms, fungi and plants, erythrose-4-phosphate condenses with phosphoenolpyruvate (PEP) from glycolysis to form chorismate, i.e. the first substrate for the shikimic pathway which leads to the production of aromatic amino acids and many aromatic

Received: 3 March 2010
Accepted: 14 December 2010

secondary metabolites, such as flavonoids, indole acetate, UV light protectants and lignin (Hermann & Weaver, 1999). These metabolites play an important role in the interaction of plant with the environment. However, its contribution to carbon metabolism in green plants is difficult to assess as plants have dual compartmentations.

The OPPP operates in the cytoplasm of bacteria, cyanobacteria, yeast and animals. Many studies have focused on the determination of the subcellular compartmentation of the OPPP enzymes in plants, such as measurement of the enzymatic activities, and measurement of the fluxes through the OPPP enzymes by feeding with radio-labelled substrates; however, the results are still controversial and uncertain due to the limitations of each technique. Several database networks (e.g. www.genome.jp/kegg/genes.html; www.tigr.org; http://mips.gsf.de; www.arabidopsis.org and www.ncbi.nlm.nih/gov) reveal that most the OPPP enzymes are present in both the cytoplasm and the plastid, except possibly TAL and TK, which are thought to be plastid localized. The latter would result in an incomplete OPPP in the cytosol of the plant cell. Thus, it can be concluded that there is no genetic evidence for a complete OPPP in the cytosol of plant to date. The aim of this study was to over-express *E. coli TAL* in the cytosol of *A. thaliana* Col-0 in order to produce transgenic plants which have a complete OPPP in the cytosol. Two genes encoding distinct isoforms of *E. coli* TAL, *talA* (GenBank accession number P0A867) and *talB* gene (GenBank accession number P0A870) were used in this study to over-express in *A. thaliana*.

MATERIALS AND METHODS

Preparation of the Plant Material for Transformation

A. thaliana ecotype Columbia (Col-0) was grown in the controlled-environment growth cabinet in the Central Annexe Facilities, University of Sheffield. The growth cabinet was set at 20°C and an irradiance of photosynthetic photon flux density (PPFD) of 250 $\mu molm^{-2}s^{-1}$. Meanwhile, photoperiod was set for 14 hours for transformation and seed production.

Constructions of E. coli *TAL to Over-express in Cytosol*

E. coli talA and *talB* (obtained from Dr. Sachiko, University of Sheffield) were ligated in the sense orientation into the *XbaI* and *SstI* sites of the pMOG22. The ligations were then sub-cloned into subcloning efficiency DH5α competent cells (Gibco). The transformed *E. coli* were spread on the LB (Luria-Bertani) plates (5.0 g NaCl, 10.0 g Bacto Tryptone, 5.0 g Bacto Yeast Extract, 15 g of Bacto-Agar, 100 µl of 10 N NaOH and ddH_20) containing antibiotic kanamycin (50 mg/L). Successful transformants were identified by the formation of colonies on the media. The plasmid vectors were purified from positive colonies by minipreps and the isolated plasmids were further analysed by agarose gel electrophoresis.

Construction of Agrobacterium *Electro-competent Cells*

Agrobacterium tumefaciens (C58) was grown in 50 ml of YMB medium (0.5 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4.7H_2O$, 0.1 g L^{-1} NaCl, 10 g L^{-1} mannitol, 0.4 g L^{-1} yeast extract and pH 7.0, HCl) with 200 mg L^{-1} rifampicin for 2 days at 28ºC with shaking. The culture was kept cooled on ice for 30 min and then centrifuged at 4,000×g at 4ºC for 10 min. The pellet was resuspended in 1 ml of 10% (v/v) glycerol.

Electroporation of Agrobacterium

E. coli TAL constructs were inserted into *Agrobacterium* through electroporation, in which 100 µl of *A. tumefaciens* and 2 µl of each constructs were electroporated by using Bio-Rad GenePulser (Miller & Nickoloff, 1995).

Arabidopsis Transformation by *Agrobacterium* Mediated Method

Preparation of the Agrobacterium Cultures

Agrobacterium tumefaciens, C58, was grown in 10 ml YMB cultures with 50 mg/L hygromycin at 28°C for 2 days. 1 ml of the cultures was inoculated into 250 ml of YMB cultures with 50 mg/L hygromycin and incubated at 28°C for 2 days. The cultures were then centrifuged at 3,000 x g for 10 min at 4°C, and the pellets were resuspended in 5% sucrose to give the OD at 600nm, OD_{600}=0.8-1.5 before 0.05% Silwet was finally added prior to dipping plants.

Transformation

Arabidopsis was transformed by immersion of inflorescences in a suspension of *A. tumefaciens* solution for 5-10 seconds with gentle agitation (Clough & Bent, 1998). The plants were then covered with black plastic overnight. It is important to note that these plants were watered as usual and this was stopped once the seeds became mature. All self-fertilized seeds were collected to screen transgenic lines.

Screening of the Transgenic Lines

Sterilisation of Seeds and Selection of Hygromycin-Resistant Transformants

Seeds were sterilized with 50% (v/v) ethanol and left at room temperature for 10 min prior to centrifugation at 10,000 rpm for 30 sec. The seeds were then washed with 50% (v/v) bleach and vortex vigorously, left for 5-10 min at room temperature and centrifuged at 10,000 rpm for 30 sec. These seeds were then rinsed four times with sterile distilled water and dried under sterile condition. Later, the sterilized seeds were plated onto the MS media with 200 mg/L hygromycin and grown for 2 weeks in the growth chamber (Versatile Environmental Test Chamber, Sanyo) at 20°C in the light (200 µmol quanta $m^{-2}s^{-1}$) with 10 hours of photoperiod.

Small Scale DNA Extraction from Plant Material for the PCR Analysis

Small pieces of leaf samples were taken from the plants at week 3. Pre-heated (65°C) DNA extraction buffer (200 mM Tris, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added and ground using a mixer mill for 5 min. The samples were incubated in the water bath at 65°C for 15 min, followed by centrifuging at 14,000 rpm for 10 min. Supernatant was transferred into a 1.5 ml tube containing isopropanol with 1:1 ratio and mixed by inverting the tube several times. The DNA was precipitated at room temperature for 15 min. The samples were then centrifuged at 14,000 rpm for 15 min and the supernatant was discarded. The pellet was washed twice with 50 µl of 70% ethanol and air-dried. Finally, the pellet was resuspended in 50µl of sterile distilled water and stored at -20°C until subsequent molecular analysis.

Measurement of the Transaldolase Activity

Supernatant (as prepared above) was desalted with PD10 column. A solution (1ml) containing 50 mM HEPES-HCl, pH 8.5, 20 mM NADH, 20 mM F6P and 0.6U/1.8U glycerol 3-phosphate dehydrogenase/triose phosphate isomerase enzyme mixture (G3PDH/TPI) and desalted supernatant was measured spectrophotometrically (Ultrospec 2000, Pharmacia Biotech, UK) at 340 nm to quantify transaldolase activity (Brand, 1983; Schnarrenberger *et al*., 1995; Sprenger *et al.*, 1995).

Immunoblotting

Preparation of the Samples

Sample buffer (62.5 mM Tris-HCl, pH 6.8, 20 % [v/v] glycerol, 10 % SDS, 5 % [v/v] β-mercaptoethanol, 0.012 % [v/v] bromophenol blue) was added to the supernatant (as prepared above) in the ratio of 1:1 ratio before it was boiled for 5 min.

Preparation of Gels and Electrophoresis

Proteins were separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) by using Mini-Protean 3 cell (Bio-Rad). The gels consist of upper 5 % stacking gel (5.7 ml ddH_2O, 1.7 ml 30% degassed Acrylamide/Bis, 2.5 ml gel buffer 0.5 M Tris-HCl pH 6.8, 100 µl of freshly prepared 10 % (w/v) SDS, 50 µl 10% (w/v) APS and 10 µl NNN'N'-tetramethylethylenediamine (TEMED) and lower 10% running/resolving gel (3.4 ml ddH2O, 4.0 ml 30% degassed Acrylamide/Bis, 2.5 ml gel buffer 1.5 M Tris-HCl pH 8.8, 100 µl 10% (w/v) SDS, 50 µl of the freshly prepared 10 % (w/v) APS and 5 µl TEMED).

Immunoblotting

Protein samples (15 µg) were loaded on the gel and run at 200 V for an hour (Bio-Rad, USA). The proteins were then transferred to Immobilon-P polyvinylidene difluoride membranes (PDVF) (Millipore, Bedford, UK) at 30 V for overnight membranes using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad), according to the manufacturer's instruction. After the transfer, the membranes were washed in TBS (20 mM Tris-HCl, pH7.4, 140mM NaCl) by shaking for them for 2-5 min at room temperature. The membranes were blocked with 3% milk/TBS for 1 hour on the orbital shaker and washed four times with TBS for 5 min. The membranes were then probed with *talA* or *talB* antibodies (obtained from Dr. Sachiko, University of Sheffield) for 1 hour on the orbital shaker. The primary antibodies were prepared as a 1:1000 dilution in 3% milk/TBS. The membranes were washed four times with TBS for 5 min and subsequently probed with secondary antibodies for 1 hour on the orbital shaker, after which time they were washed with TBS for 5 min, and this washing step was repeated four times. The secondary antibodies conjugated with peroxidase were prepared as a 1:1000 dilution in 3% milk/TBS. Finally, the protein bands were visualized using an ECL chemiluminescence detection reagent kit (Amersham Life Sciences Limited, Buckinghamshire, UK). The band image was captured on Kodak BioMax MR film (Kodak, New York, USA).

RESULTS AND DISCUSSION

Construction of the Plant Transformation Vectors

This study used prokaryotic TAL from *E. coli*, encoded by *talA* and *talB* genes (obtained from Dr. Sachiko Shimizu, University of Sheffield) in order to avoid gene silencing of the endogenous gene. The introduction of a homologous gene expressed constitutively in the plant cell can often result in the silencing of the endogenous gene via co-suppression (Primrose *et al.*, 1996). Therefore, *E. coli* TAL is suitable to be over-expressed in the plants because it has low homology to plant genes of about 43% to 49% (Caillau, 2002). The suitability of *E. coli* TAL to be over-expressed in plants had been proven by Shimizu (2002), based on the kinetic properties of *E. coli TAL*, which has an optimum pH 8.5-9.5, while the optimum pH of recombinant transaldolase of tomato is 8.3-8.8 (Caillau, 2002). Thus, *E. coli* TAL will be active when they are over-expressed in plants because they have similarities in pH optima. The Km value of F6P and E4P of *E. coli* TAL are 1.2 mM and 0.09 mM, while the Km value of F6P and E4P of spinach are 1.0 mM and 0.05 mM (Tsolas & Horecker, 1972) and the recombinant transaldolase of tomato are 0.35 mM and 0.13 mM (Caillau, 2002). Therefore, a range of the Km value of F6P and E4P of *E. coli TAL* and plant TAL is similar, which is 0.35 to 1.20 mM for F6P and this is 0.05 to 0.13 mM for E4P. Thus, it is assumed that the concentrations of F6P and E4P are high enough to drive the maximum activity of TAL in the cytosol.

Plant transformation vectors were constructed for the expression of *E. coli* TAL in the cytosol of *A. thaliana*. *E. coli talA* and *talB* were ligated in the sense orientation into the *XbaI* and *SstI* sites of the pMOG22 backbone (*Fig. 1a*). Figure 1b shows that the construction

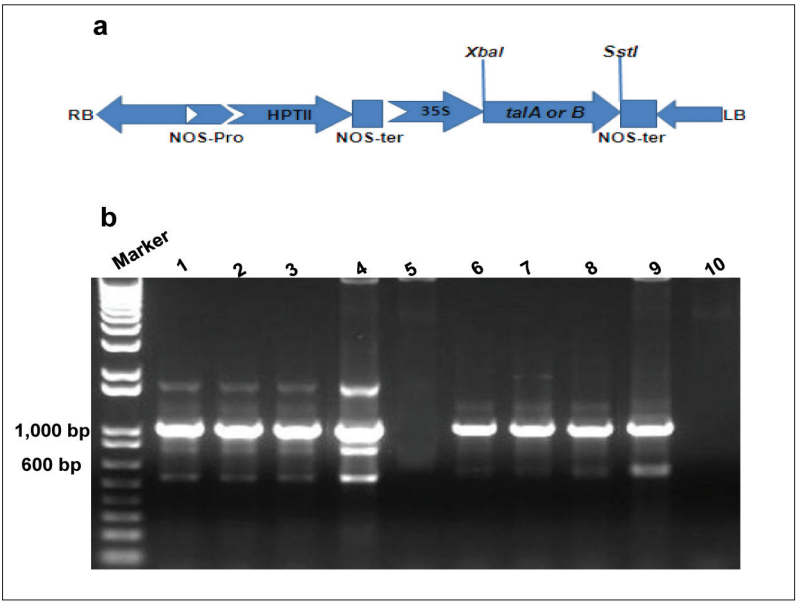

*Fig. 1: The structure of plant transformation vector pMOG22-35S-*talA *or* talB *and PCR analysis of pMOG22-35S-*talA *and* talB. *a) T-DNA region containing the Hygromycin gene (HPTII) as a selectable marker under the control of the nopaline synthase promoter (NOS-pro), and followed by NOS-ter.* E. coli talA *or* talB *were inserted in the sense orientation between XbaI and SstI restriction sites. The expression of both TAL genes is driven by the CaMV 35S promoter. RB: right border; LB: left border of T-DNA. b) clones were subjected to PCR to ensure that ligation reaction was successful. Lanes 1-4 show bacterial colonies containing* talA *gene. Lanes 6-9 show bacterial colonies containing* talB *gene. Lanes 5 and 10: negative control and empty pMOG22 binary vector*

of plant transformation vectors was successful. Then, the constructs of *talA* and *talB* plasmids were electroporated into *Agrobacterium* electro-competent cells.

Introduction of E. coli *TAL into* A. thaliana *via* Agrobacterium*-mediated Transformation Method*

Fig. 2a shows that transgenic plants were clearly identified as they produced green and healthy secondary leaves. Roots deeply invaded into a medium containing antibiotic hygromycin. Polymerase Chain Reaction (PCR) analysis showed that the putative transgenic plants could be identified by specific amplification of a 600 bp product corresponding to the *E. coli* TAL on the agarose gel (*Fig. 2b*).

The expression of *E. coli* TAL in *Arabidopsis* was further analyzed by immunoblotting and measurement of transaldolase activity. The protein extract from the transgenic plants showed a strong immuno-reaction with antibodies against *talA* or *talB*. The transaldolase activity of transgenic plants is about 2 to 6-folds higher than that of the wild type plants. These results also showed that the levels of expression of *E. coli talA* and *talB* varied among the transgenic plants (Figure 3a). Immunoblot band intensity of the transgenic plants was found to be positively correlated with transaldolase activity, which is *talA* transgenic lines ($r=0.732$, $p<0.01$) and in *talB* transgenic lines ($r=0.975$, $p<0.01$). This indicated that both over-expressed of TAL protein were functional in transgenic lines.

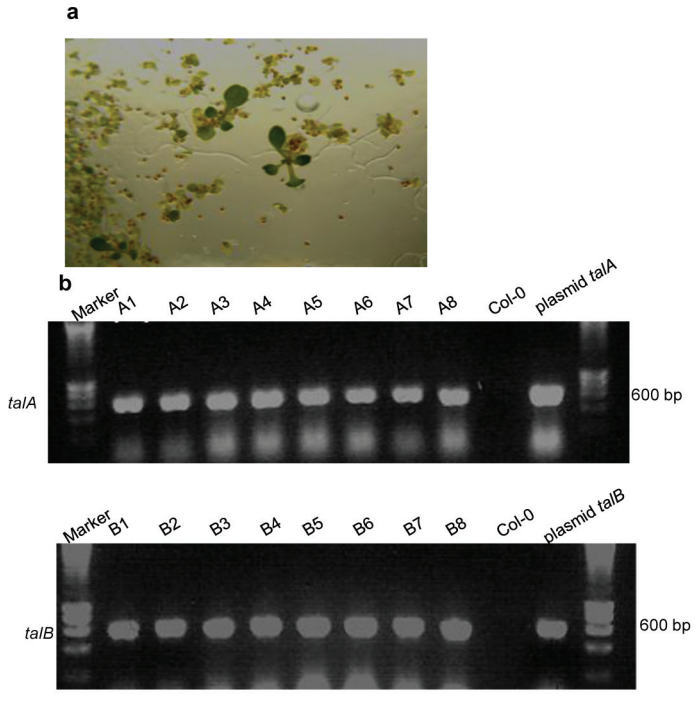

Fig. 2: Screening of transgenic Arabidopsis *plants. a, Seedlings showing resistance to hygromycin were identified as transgenic plants. b) PCR screening of transgenic plants. Col-0:*A. thaliana *Col-0 (wild type)*

Before carrying any further analysis, it was therefore preferable to ascertain the number of transgene in the range of TAL lines produced. Transgenic plants having a single copy of the transgene are desirable since they show more uniformity and transgene expression is likely to be more stable in subsequent generations (Windels *et al.*, 2003; Peach & Velten, 1991; Tang *et al.*, 2007). The seeds of the self-pollinated T_1 plants were plated onto the medium containing hygromycin. The progeny of T_1 plants (T_2 lines) showing a 3 to 1 ratio was selected for further analysis by counting the number of hygromycin resistant and sensitive seedlings about 2-3 weeks after germination. Three out of 8 T_1 seedlings of *talA* and *talB* transgenic lines showed a 3 to 1 segregation, which were lines A3, A4, A6**,** B5, B6 and B8. These seedlings were transferred to pots containing compost for further analysis. Immunoblotting and transaldolase activity were verified again in T_2 plants to ensure that the over-expressed *E. coli* TAL was inherited and stable in T_2 generation. *Fig. 4* shows strong immuno-reactions between protein extract and antibodies against *E. coli talA* or *talB,* which ensure that T_2 plants inherit normally the expression of *E. coli* TAL from T_1 and also confirm that the expression of *E. coli* TAL is stable in T_2 plants. The total transaldolase activity in T_2 transgenic plants is about 3 to 11 folds higher than that of the wild type. Similarly, the transgenic lines also showed positive correlations between band intensity and total transaldolase activity ($r=0.895$, $p<0.01$ in *talA* plants and $r=0.796$, $p<0.01$ in *talB* plants) (*Fig. 4*), suggesting that both expressed proteins were functional.

Selected T_2 independent lines with single TAL insertion were grown to produce seed, while T_3 homozygous lines were identified

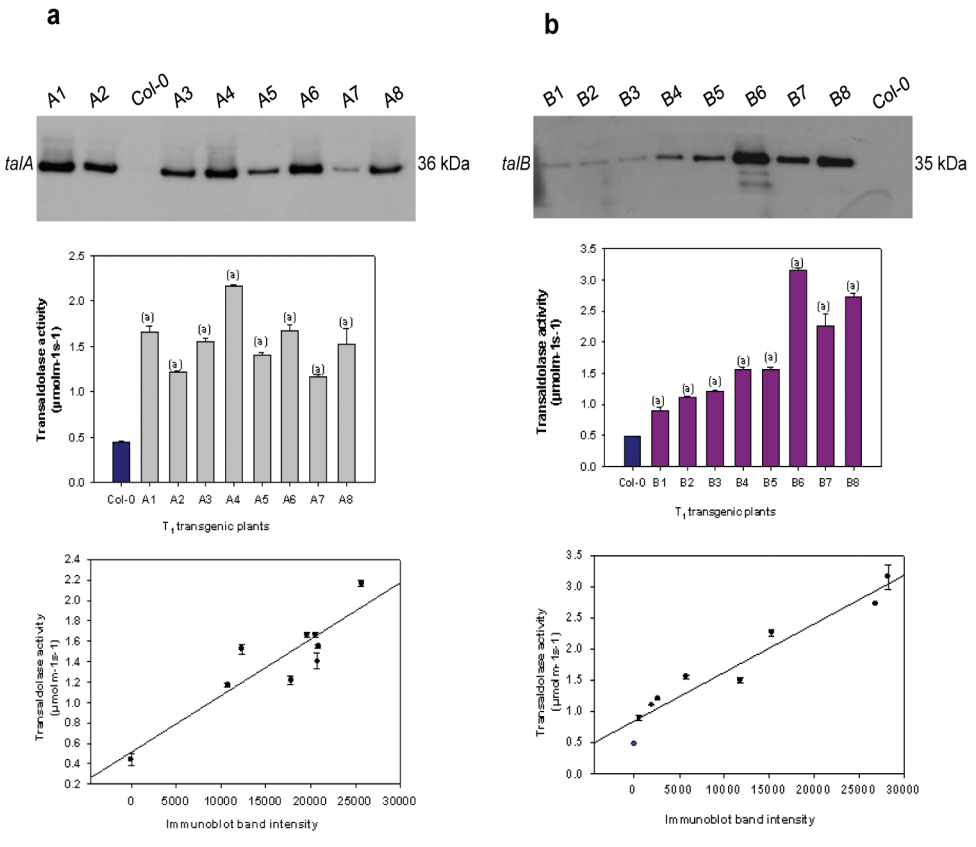

Fig. 3: Immunoblotting, total transaldolase activity and the correlation between total transaldolase activity and immunoblot band intensity of putative T_1 transgenic plants; (a) Putative T_1 transgenic plants over-expressing E. coli talA *in the cytosol; (b) Putative T_1 transgenic plants over-expressing* E. coli talB *in the cytosol. The activity was measured at Week 6 after planting. Values representing the mean and standard errors of the assays were derived from at least three replications [ANOVA and Tukey multiple comparison test ($p<0.05$) and labelled with the letter code (a)]*

after screening on the selective plate (100 % germination). A total 10 of 13 independent homozygous lines were obtained from the batch of the plants transformed with the *E. coli talA* over-expression construct (lines A3-1, A3-2, A3-6, A4-1, A4-3, A4-4, A4-5, A4-6, A6-1, A6-2, A6-3, A6-4 and A6-6). Similarly, 10 independent single insertion and homozygous lines were obtained from the batch of the plants transformed with the *E. coli talB* over-expression construct (lines B5-5, B5-6, B5-7, B6-1, B6-5, B6-6, B8-1, B8-2, B8-6 and B8-7). All of the transgenic lines looked similar to the wild type plants and did not appear to have any distinct visible phenotype. In particular, the height of the plant is similar to that of the wild type, while flowering time and the leaves started senescing at about the same week as the wild type (*Fig. 4c*).

CONCLUSION

Over-expression of *E. coli* TAL in the cytosol of *A. thaliana* was successful. The expression of *E. coli* TAL was stable and functional from

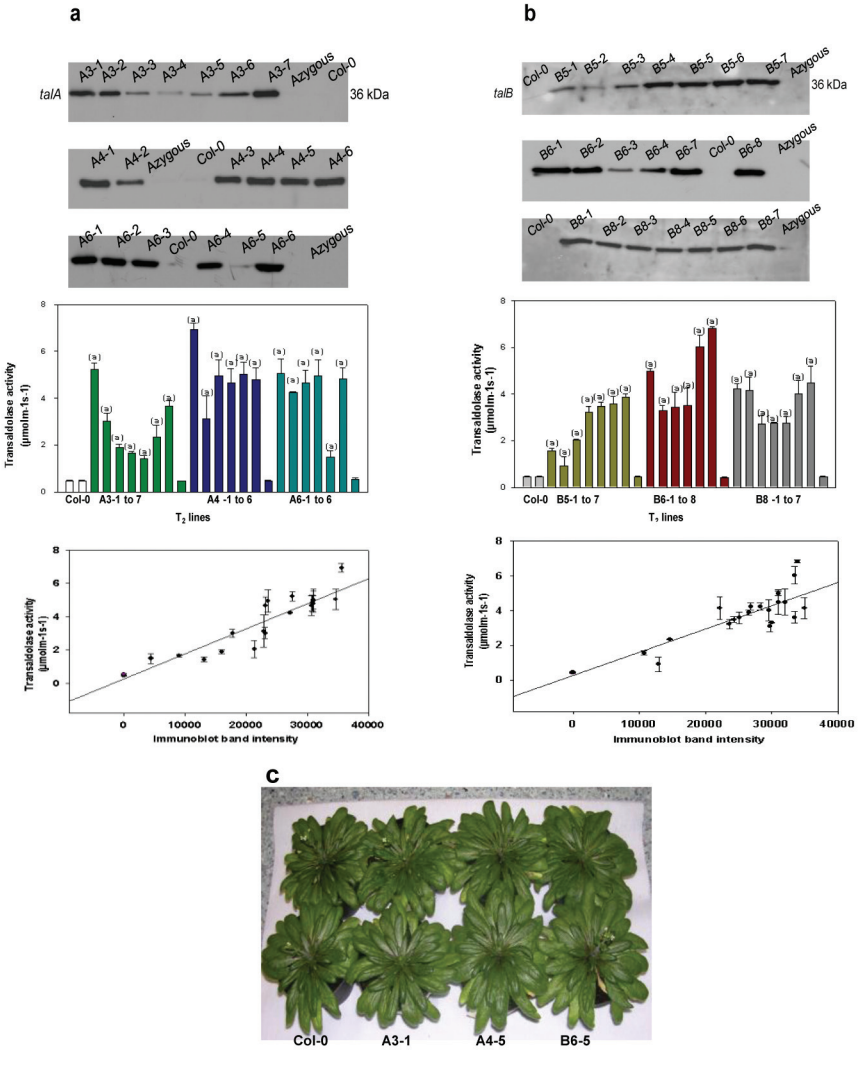

Fig. 4: Immunoblotting, total transaldolase activity, the correlation between total transaldolase activity and immunoblot band intensity and the phenotype of T_2 transgenic lines of E. coli *TAL over-expressed in the cytosol; (a) T_2 lines of* E. coli talA *over-expressed in the cytosol; (b) T_2 lines of* E. coli talB *over-expressed in the cytosol.* A. thaliana *Col-0 (wild type) and azygous lines did not react with the antibody. The activity was measured at Week 6 after planting. Values representing the mean and standard error of assays were derived from at least three replications [ANOVA and Tukey multiple comparison test ($p<0.05$) and labelled with the letter code (a)]; (c) the phenotype of transgenic lines*

T_1 to T_2 generations. Nonetheless, a complete compartmentation in the cytosol of plant did not affect plant phenotype.

ACKNOWLEDGEMENTS

The authors wish to thank the Department of Public Service, Malaysia for the financial support, as well as Prof. Dr. Paul Quick and Dr. Maxime Caillau for the technical support. A special appreciation also goes to Dr. Sachiko Shimizu for *E. coli talA* and *talB* and their antibodies.

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