In vivo Evaluation of Transgenic Watercress Containing Gene Encoding Escherichia coli Heat-Labile Toxin B Subunit

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ORIGINAL ARTICLE

In vivo evaluation of transgenic watercress containing gene encoding *Escherichia coli* heat-labile toxin B subunit

Nguyen Hoang Loc · Nguyen Van Song · Dang Thanh Long · Tae-Geum Kim · Moon-Sik Yang

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Abstract In this work, the characterizations of the LTB (*Escherichia coli* heat-labile toxin B subunit) transgenic watercresses through *Agrobacterium tumefaciens*-mediated transformation (A1 and A3-A5) and by using biolistic method (B1 and B4) were investigated. Generally, their growth is not remarkably different from the wild-type. Their physiological and biochemical characteristics are relatively different in which plant A1 has highest values such as pigment (0.92 mg g^{-1}), photosynthetic rate (23.39 mgCO_2 $[dm^2]^{-1}h^{-1}$), dry matter (7.42 %), vitamin C (0.34 mg g^{-1}), calcium (0.83 mg g^{-1}), and potassium (2.47 mg g^{-1}). The dry matter and calcium of all the transgenic watercresses and the wild-type are the same content. Southern blot hybridization showed the transgenic plants contain 1–2 copies in the genome. LTB protein strongly expresses in all the transgenic plants with contents from 1.16 to 1.46 % of total soluble protein. The GM1-ELISA binding assay indicated that plant-derived LTB protein bound to GM1-ganglioside receptors.

Keywords Growth · LTB · *Nasturtium officinale* · Physiological and biochemical characteristics

N. H. Loc (✉) · D. T. Long
Centre for Technology Incubation and Transfer,
Hue University, Hue 47000, Vietnam
e-mail: nhloc@hueuni.edu.vn

N. V. Song
National Technical College of Medicine No II, Danang, Vietnam

T.-G. Kim · M.-S. Yang
Division of Biological Sciences and Research Center of Bioactive Materials, Chonbuk National University, Jeonju, Chonbuk 561-756, South Korea

Abbreviations

BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	Bovine serum albumin
CTB	Cholera toxin B subunit
dUTP	2′-deoxyuridine, 5′-triphosphate
ELISA	Enzyme-linked immunosorbent assay
ETEC	Enterotoxigenic *Escherichia coli*
GM1	Monosialotetrahexosyl ganglioside
LTB	*Escherichia coli* heat-labile enterotoxin B subunit
NBT	Nitro blue tetrazolium
TSP	Total soluble protein

Introduction

Watercress (*Nasturtium officinale* L.), member of the Brassicaceae family, is fast-growing, aquatic or semi-aquatic, and perennial plants. It was grown in many parts of the world and is one of the oldest green vegetables (Phillips and Rix 1995; Jin et al. 1999). Watercress contains many vitamins and minerals, and has long been valued as a food and medicinal plant. Considered a cleansing herb, its high vitamin C content makes it a particularly valuable remedy for chronic illness (Chevallier 1996).

The LTB of ETEC is known to be a potent mucosal adjuvant (Tochikubo and Yasuda 2000; Kang et al. 2003). It is considered a subunit vaccine candidate to be used against ETEC-induced diarrhea (Rezaee et al. 2005). There were several reports on the introduction of gene into watercress, e.g. Jin et al. (1999) successfully transferred the *Bt, cry*IIa3 and *npt*II genes into watercress through *Agrobacterium*-mediated transformation. Li et al. (2000) also transferred a gene encoding for betaine-aldehyde dehydrogenase (BADH) into watercress. Ogita et al. (2009) studied simple shoot

Table 1 Growth of LTB-transgenic watercresses (42-days old)

Plants	Plant height (cm)	Leaf number	Leaf area (cm^2)	Root number	Root lenght (cm)
Wild-type	15.61^{ab}	13.30^{a}	2.20^{a}	1.30^{a}	3.56^{a}
A1	15.74^{a}	13.40^{a}	2.14^{ab}	1.23^{a}	3.51^{a}
A3	15.71^{a}	13.10^{a}	2.19^{a}	1.20^{a}	3.54^{a}
A4	15.65^{a}	13.33^{a}	2.21^{a}	1.16^{a}	3.43^{ab}
A5	14.66^{d}	12.06^{b}	2.05^{bc}	1.16^{a}	3.36^{ab}
B1	15.13^{bcd}	11.93^{b}	2.03^{c}	1.16^{a}	3.31^{ab}
B4	14.92^{dc}	11.63^{bc}	2.07^{bc}	1.16^{a}	3.21^{b}

Different letters in a column indicate significantly different means (Duncan's test, $p < 0.05$)

multiplication procedure using internode explants, and its application for particle bombardment and *Agrobacterium*-mediated transformation in watercress. The growth and development of transgenic plants under in vivo condition to be also evaluated by Lieman-Hurwitz et al. (2003), Chen and Xu (2007), Wang et al. (2012), Kiełkiewicz et al. (2012), Reinecke et al. (2013).

The purpose of this study is to characterize four LTB transgenic watercresses through *Agrobacterium tumefaciens*-mediated transformation (A1 and A3-A5) and two others by using biolistic method (B1 and B4) grown under natural condition, to compare physiological and biochemical properties, and their LTB protein productivity.

Materials and methods

Plant materials

Transgenic watercresses (*Nasturtium officinale* L.) developed with the construct of pMYO51 vector for LTB expression (Kang et al. 2004). Four plants (#1, #3, #4 and #5 were renamed A1, A3, A4 and A5) were transferred the synthetic LTB gene through *Agrobacterium tumefaciens*-mediated transformation (Loc et al. 2011), the others (#1 and #4 were renamed B1 and B4) by using biolistic method (Loc et al. 2010b). Transgenic plants were maintained in in vitro condition on the MS (Murashige and Skoog 1962) medium supplemented with 3 % sucrose, 0.8 % agar and 1 mg l^{-1} napthaleneacetic acid. Plantlets with developing root systems were transferred to a plastic tray in sandy soil, coconut fiber and bio-organic fertilizer (2:1:2) mixture for evaluating the growth and development.

Physiological and biochemical analysis

Dry matter is determined by the absolute drying method, leaf area is determined through the weighing method, pigment content is measured by the colorimetric method of Weststein, and photosynthetic rate is measured by the titration of Tiurin (Grodzinxki and Grodzinxki 1981).

Cellulose content is determined by the acetic acid-nitric acid method and vitamin C content is determined through the iodine titration method (Mui 2001). Calcium and potassium contents are measured by the flame photometry method (Khoa 2000).

Biomolecular analysis

Southern blot hybridization is performed as described in Sambrook and Russell (2001). Aliquots (10 μg) of total genomic DNA from the leaves of the transgenic plant and the wild-type were digested with *Kpn* I and electrophoresed on a 0.8 % agarose gel, which was then transferred to Hybond-N+ membrane (Amersham). The blot was hybridized with a probe (synthetic LTB gene, 414 bp) labeled with digoxigenin-

Fig. 1 LTB transgenic watercress plants 42 days after planting. Wt: wild-type plants. A1, A3, A4 and A5: transgenic plants through *Agrobacterium*-mediated transformation. B1 and B4: transgenic plants by biolistic method

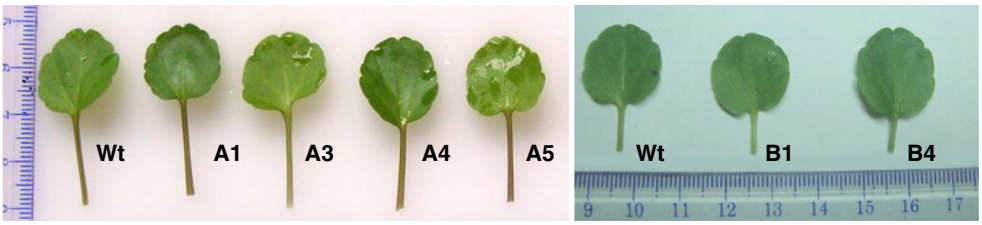

Fig. 2 Leaves of LTB transgenic watercress plants 42 days after planting. Wt: wild-type plants. A1, A3, A4 and A5: transgenic plants through *Agrobacterium*-mediated transformation. B1 and B4: transgenic plants by biolistic method

dUTP using a DNA labeling system (DIG high prime DNA labeling and detection starter kit I, Roche) at 46 °C in a hybridization incubator. After hybridization overnight, blots were washed and incubated with a ratio of antibody conjugated digoxigenin to alkaline phosphatase (1:5000 v/v) at room temperature for 30 min. The BCIP/NBT substrate is used for color development.

Western blot, ELISA and GM1-ELISA binding analyses for LTB protein are performed similarly as previous reports on LTB expression in in vitro watercress (Loc et al. 2010b, 2011), except optical density was measured at 490 nm. Total soluble protein content is determined by the method of Bradford (1976) using bovine serum albumin as a standard. The sample was read at wave length of 595 nm against the blank.

Statistical analysis

All experiments are conducted with a minimum of ten replicates and all experiments were repeated three times. The data were analyzed as means ± standard error followed by comparisons of the mean by Duncan's test ($p < 0.05$) using the SAS program.

Results and discussion

Physiological and biochemical characteristics

The growth of LTB transgenic watercresses was showed in Table 1 and Figs. 1, 2 and 3. Generally, there were no remarkable differences between the transgenic plants and the wild-type. Statistical analyses showed that root number and length of the transgenic plants are similar with the wild-type (1.16 to 1.23 vs 1.3 and 3.21 to 3.54 cm vs 3.56 cm, respectively). However, other characteristics such as plant height (14.66 to 15.74 cm vs 15.61 cm), leaf number (11.63 to 13.40 vs 13.30) and leaf area (2.03 to 2.21 cm^2 vs 2.20 cm^2) are slight differences.

Except four plants A3, A5, B1 and B4 showed lower photosynthetic rates (19.06, 19.06, 17.33 and 18.19 $mgCO_2$ $[dm^2]^{-1}h^{-1}$, respectively). Generally, other physiological and biochemical characteristics are not different between the LTB-transgenic watercresses and the wild-type (Table 2). The pigment content in the transgenic plants ranges from 0.84 to 0.92 mg g^{-1} (wild-type is 0.92 mg g^{-1}), dry matter 6.88 to 7.42 % (7.33 %), cellulose 1.89 to 2.39 % (2.36 %), vitamin C 0.31 to 0.34 mg g^{-1} (0.34 mg g^{-1}), calcium 0.81 to 0.87 mg g^{-1} (0.82 mg g^{-1}), and potassium 2.39 to 2.64 mg g^{-1} (2.53 mg g^{-1}).

In an early report, we also found non-significant differences of physiological and biochemical characteristics between LTB transgenic tomato and the wild-type grown under in vivo condition (Loc et al. 2012). Thinh and Long (2011) also obtained the same results in CTB transgenic tomato.

Lieman-Hurwitz et al. (2003) showed *ictB* transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* plants, a gene involved in HCO_3^- accumulation in cyanobacteria, exhibited significantly faster photosynthetic rates than the wild-types under limited conditions. Under conditions of low relative

Fig. 3 LTB transgenic watercress plants grown under in vivo condition. Wt: wild-type plants, A1: transgenic plants through *Agrobacterium*-mediated transformation, B1: transgenic plants by biolistic method

Table 2 Physiological and biochemical characteristics of LTB-transgenic watercress (42-days old)

Plants	Pigment (mg g^{-1})	Photosynthesis ($mgCO_2$ $[dm^2]^{-1}h^{-1}$)	Dry matter (%)	Cellulose (%)	VitC (mg g^{-1})	Calcium (mg g^{-1})	Potassium (mg g^{-1})
Wild-type	0.92^{a}	22.53^{ab}	7.33^{a}	2.36^{a}	0.34^{a}	0.82^{a}	2.53^{a}
A1	0.92^{a}	23.39^{a}	7.42^{a}	1.96^{bc}	0.34^{a}	0.83^{a}	2.47^{a}
A3	0.92^{a}	19.06^{abc}	6.88^{a}	2.39^{a}	0.32^{bc}	0.81^{a}	2.39^{ab}
A4	0.85^{c}	22.53^{ab}	7.34^{a}	2.08^{abc}	0.33^{ab}	0.85^{a}	2.64^{a}
A5	0.84^{c}	19.06^{abc}	7.30^{a}	1.89^{c}	0.31^{c}	0.84^{a}	2.41^{ab}
B1	0.84^{c}	17.33^{c}	7.19^{a}	2.10^{abc}	0.31^{c}	0.87^{a}	2.61^{a}
B4	0.85^{c}	18.19^{bc}	6.92^{a}	2.25^{ab}	0.33^{ab}	0.86^{a}	2.46^{a}

humidity, the growth of the transgenic *A. thaliana* plants was considerably faster than the wild-types. There was no difference in the amount of ribulose 1,5-bisphosphate carboxylase/oxygenase detected in the wild-types and their respective transgenic plants. The CO_2 compensation point in the *ictB*-transgenic plants was lower than in the wild-types.

According to Chen and Xu (2007), in comparison with the wild type, the silenced *OsBP-73* transgenic rice had significantly lower plant height, grain number per panicle, and some physiological and biochemical characteristics (e.g. chlorophyll, leaf net photosynthetic rate, photosynthetic electron transport and photophosphorylation rates, ribulose 1,5-bisphosphate carboxylase/oxygenase, sucrose phosphate synthase activity…); but higher intercellular CO_2 concentration, and sucrose, fructose and glycerate 3-phosphate contents.

Molecular characteristics

To investigate the integration of transgenes into the host watercress genome, further Southern blotting analysis of genomic DNA from the transgenic plants was performed. Total DNA from the transgenic plants and the wild-type plant were digested with *Kpn* I (no recognition site in the gene), and the blot was hybridized with the LTB gene used as a probe. The distribution of copy numbers obtained is given in Fig. 4. The transgenes showed the bands from about 1.5 to 3.9 kb, reflecting the presence of the LTB gene in which plants A1 and A5 have two bands (two copies), and other plants have only one band (one copy). One band can be regarded as a single insertion, but may contain a number of copies present as concatenates or incomplete copies (Yao et al. 2006). The results of hybridization showed LTB gene was integrated into the genome of transgenes. The hybridization has not been found in the wild-type plant. Jin et al. (1999) reported transformation of *ctyIIa3* gene into watercress. Southern blot analysis showed many copies present in the genome. Chikwamba et al. (2002) also found many copies of LTB gene in the genome of maize.

Western blot analysis showed all the transgenic watercresses strongly expressed the LTB protein with a molecular weight of about 55 kDa (Fig. 5). In this work, the pentamer structure of the plant-derived LTB protein had slightly higher molecular weight than that of the bacterial LTB protein (45 kDa). However, our previous studies in tobacco (Kang et al. 2003), tomato (Nhi et al. 2010), *Peperomia pellucida* (Loc et al. 2010a), even in in vitro watercress (Loc et al. 2010b and 2011) indicated that plant-derived LTB protein had molecular weight of 45 kDa equal that of bacteria. This discrepancy was presumably due to the extra six amino acids that were added at the C-terminus for endoplasmic reticulum retention (Kang et al. 2004), and the failure of the plant cells to remove the leader signal peptide (Arakawa et al. 1997).

An ELISA was used to determine the levels of the LTB protein in the leaf tissues of the transgenic plants. The amount of LTB was expressed as the percentage of total soluble protein (TSP) in the sample (Fig. 6a). ELISA results indicated that the content of the LTB proteins reached values of 1.04 to 1.46 % of TSP, it is higher than that of in vitro growing transgenic watercresses (0.85 to 1.3 % of the TSP) (Loc et al. 2011).

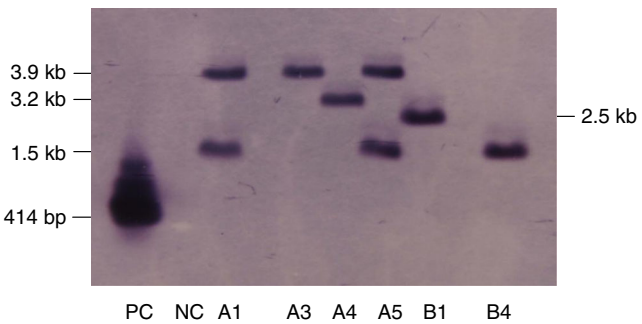

Fig. 4 Southern blot analysis. PC: LTB gene. NC: wild-type plant. A1, A3, A4 and A5: transgenic plants through *Agrobacterium*-mediated transformation. B1 and B4: transgenic plants by biolistic method

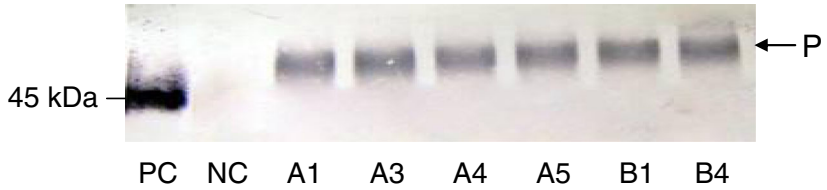

Fig. 5 Western blot analysis. PC: bacterial LTB protein. NC: wild-type plant. A1, A3, A4 and A5: transgenic plants through *Agrobacterium*-mediated transformation. B1 and B4: transgenic plants by biolistic method. P: pentamer structure of plant-derived LTB protein with a molecular weight of about 55 kDa

The ability of the LTB protein to bind to GM1-gangliosides was examined using GM1-ELISA. In this assay, the plant-produced LTB protein demonstrated relative strong affinities for GM1-ganglioside (OD 490 nm values are from 0.26 to 0.34), but almost not for BSA (0.04 to 0.05) (Fig. 6b). The affinity of the LTB to GM1 indicated that binding of LTB the plant-derived protein to GM1 is co-operative. In early reports, we found the binding affinities are from 0.08 to 0.24 in the LTB transgenic watercresses transformed through *Agrobacterium* with 0.008 to 0.012 for BSA (Loc et al. 2011), and from 0.22 to 0.24 in the LTB transgenic watercresses transformed by bombardment with 0.003 to 0.007 for BSA (Loc et al. 2010b).

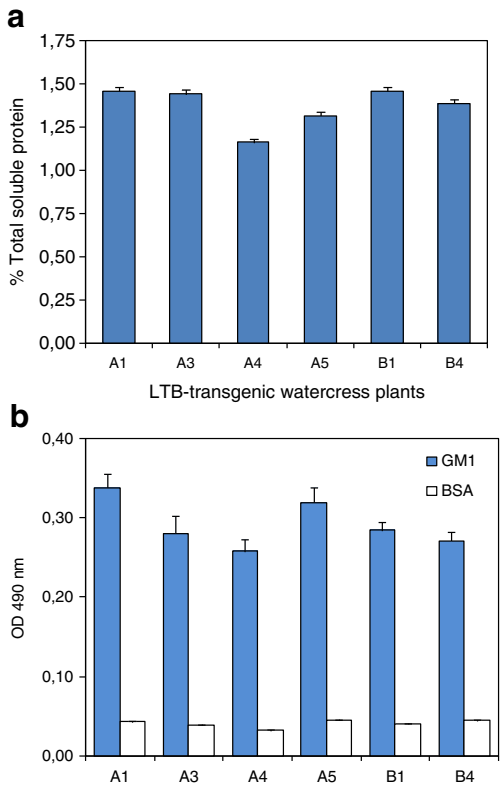

Fig. 6 Expression analysis of total soluble protein. (**a**) ELISA analysis and (**b**) GM1-ELISA analysis. A1, A3, A4 and A5: transgenic plants through *Agrobacterium*-mediated transformation; B1 and B4: transgenic plants by biolistic method

In conclusions, the evaluation for two groups of LTB-transgenic watercresses (via *Agrobacterium* transformation and by biolistic method) grown under in vivo condition showed that their growth is similar to each other and to the wild-type. Physiological and biochemical properties have slight differences. Expression levels of the LTB protein and its GM1 binding affinities of *in planta* transgenic plants are higher than that of in vitro growing transgenic plants.

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References

Arakawa T, Chong DK, Merritt JL, Langridge WH (1997) Expression of cholera toxin B subunit oligomers in transgenic potato plants. Transgenic Res 6:403–413

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Chen Y, Xu DQ (2007) Changes in leaf photosynthesis of transgenic rice with silenced *OsBP-73* gene. Photosynthetica 45:419–425

Chevallier A (1996) The encyclopedia of medicinal plants. Dorling Kindersley, London

Chikwamba R, McMurray J, Shou H, Frame B, Pegg SE, Scott P, Manson H, Wang K (2002) Expression of a synthetic *E.coli* heat-labile enterotoxin B sub-unit (LT-B) in maize. Mol Breed 10:253–265

Grodzinxki AM, Grodzinxki DM (1981) Handbook of Plant Physiology. Technology and Science Publishing House, Ha Noi (in Vietnamese)

Jin RG, Liu YB, Tabashnik BE, Borthakur D (1999) Tissue culture and *Agrobacterium*-mediated transformation of watercress. Plant Cell Tissue Organ Cult 58:171–176

Kang TJ, Loc NH, Jang MO, Jang YS, Kim YS, Seo JE, Yang MS (2003) Expression of the B subunit of *E.coli* heat-labile enterotoxin in the chloroplasts of plants and its characterization. Transgenic Res 12: 683–691

Kang TJ, Han SC, Jang MO, Kang KH, Jang YS, Yang MS (2004) Enhanced expression of B-subunit of *Escherichia coli* heat-labile enterotoxin in tobacco by optimization of coding sequence. Appl Biochem Biotechnol 117:175–87

Khoa LV (2000) Methods of analysis of soil, water, fertilizer and crop. Education Publishing House, Ha Noi (in Vietnamese)

Kiełkiewicz M, Gajc-Wolska J, Ślusarz S, Szwacka M (2012) Growth, development and yield of transgenic 35S-thaumatin II-expressing cucumber plants – open field evaluation. Sci Hortic 143:82–91

Li YX, Chang FQ, Du LQ, Guo BH, Li HJ, Zhang JS, Chen SY, Zhu ZQ (2000) Genetic transformation of watercress with a gene encoding for betaine-aldehyde dehydrogenase (BADH). J Integr Plant Biol 42:315–318

Lieman-Hurwitz J, Rachmilevitch S, Mittler R, Marcus Y, Kaplan A (2003) Enhanced photosynthesis and growth of transgenic plants that express *ictB*, a gene involved in HCO_3^- accumulation in cyanobacteria. Plant Biotechnol J 1:43–50

Loc NH, Bach NH, Kim TG, Yang MS (2010a) Tissue culture and expression of *Escherichia coli* heat-labile enterotoxin B subunit in transgenic *Peperomia pellucida*. Protein Expr Purif 72:82–86

Loc NH, Song NV, Tien NQD, Minh TT, Nga PTQ, Phuong TTB, Kim TG, Yang MS (2010b) Expression of heat-labile enterotoxin B subunit from *E. coli* in transgenic watercress. Vietnam J Biotechnol 8:1173–1179

Loc NH, Song NV, Tien NQD, Minh TT, Nga PTQ, Phuong TTB, Kim TG, Yang MS (2011) Expression of the *Escherichia coli* heat-labile enterotoxin B subunit in transgenic watercress (*Nasturtium officinale* L.). Plant Cell Tissue Organ Cult 105:39–45

Loc NH, Long DT, Nhi PY, Hong HTK, Kim TG, Yang MS (2012) Preliminary evaluation of transgenic tomato plants expressing *Escherichia coli* heat-labile toxin B subunit grown under *in vivo* condition. Ann Biol Res 3:2070–2073

Mui NV (2001) Practice in Biochemistry. Technology and Science Publishing House, Ha Noi (in Vietnamese)

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nhi PY, Kim TG, Yang MS, Loc NH (2010) Expression of *Escherichia coli* heat-labile enterotoxin B subunit in transgenic tomato (*Lycopersicon esculentum* L.). Vietnam J Biotechnol 8:1279–1285

Ogita S, Usui M, Shibutani N, Kato Y (2009) A simple shoot multiplication procedure using internode explants, and its application for particle bombardment and *Agrobacterium*-mediated transformation in watercress. J Plant Res 122:455–463

Phillips R, Rix M (1995) Vegetables. Macmillan Reference Books, London

Reinecke DM, Wickramarathna AD, Ozga JA, Kurepin LV, Jin AL, Good AG, Pharis RP (2013) Gibberellin 3-oxidase gene expression patterns influence gibberellin biosynthesis, growth, and development in pea. Plant Physiol 163(2):929–45. doi:10.1104/pp.113.225987

Rezaee MA, Rezaee A, Moazzeni SM, Salmanian AH, Yasuda Y, Tochikubo K, Pirayeh SN, Arzanlou M (2005) Expression of *Escherichia coli* heat-labile enterotoxin B subunit (LTB) in *Saccharomyces cerevisiae*. J Microbiol 43:354–360

Sambrook J, Russell DW (2001) Molecular Cloning: A Laboratory Manual, 3rd edn. Cold Spring Harbor Laboratory, Plainview

Thinh LT, Long DT (2011) Morphological and qualitative characteristics of CTB (cholera toxin B subunit) transgenic tomato fruit. J Sci Hue Univ 64:159–165

Tochikubo K, Yasuda Y (2000) Principles of mucosal immunity and development of mucosal vaccines using cholera toxin B subunit and its related adjuvants. Recent Res Dev Microbiol 4:387–405

Wang GK, Zhang M, Gong JF, Guo QF, Feng YN, Wang W (2012) Increased gibberellin contents contribute to accelerated growth and development of transgenic tobacco overexpressing a wheat ubiquitin gene. Plant Cell Rep 31:2215–27

Yao Q, Cong L, Chang JL, Li KX, Yang GX, He GY (2006) Low copy number gene transfer and stable expression in a commercial wheat cultivar via particle bombardment. J Exp Bot 57:3737–3746