

Shoot tip regeneration and optimization of *Agrobacterium tumefaciens*-mediated transformation of Broccoli (*Brassica oleracea* var. *italica*) cv. Green Marvel

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Abstract A protocol of plant regeneration from shoot tips and optimization of *Agrobacterium tumefaciens*-mediated transformation of broccoli (*Brassica oleracea* var. *italica*) cv. Green Marvel have been developed. Shoot tip response was assessed on Murashige and Skoog (MS) medium supplemented with different concentrations of zeatin. The highest regeneration with a maximum of 13 shoots per explant was obtained on MS medium containing 1.5 mg l^{-1} zeatin. Primary selection of putative transformed explants was performed on the optimized regeneration medium (MS medium containing 1.5 mg l^{-1} zeatin and 80 mg l^{-1} kanamycin) for 60 days. The effects of preculture, acetosyringone and growth of bacterial culture were studied. Explants precultured on callus induction medium for 4 days prior to inoculation with *A. tumefaciens* with $200 \text{ }\mu\text{M}$ acetosyringone resulted in improved transformation frequency. The *Agrobacterium* culture dilution of 1:5 and inoculation time of 30 min increased the efficiency of transformation of shoot tip explants. The results also indicated that 150 mg l^{-1} ampicillin alone was adequate to eradicate *Agrobacterium* growth in the SRM incorporated with the respective minimum inhibitory concentration of 80 mg l^{-1} kanamycin. The polymerase chain reaction (PCR) and Southern blot assays confirmed the transgenic status of the broccoli cv. Green Marvel regenerants. A transformation efficiency of 5 % was achieved based on the positive PCR results using the optimized

procedure. The expression of luciferase reporter gene in the transformed cells and the transcription of *AtHSP101* using RT-PCR further confirmed the transgenic status of the regenerated plants.

Keywords Broccoli · *AtHSP101* · Luciferase gene · Southern blot · *Agrobacterium tumefaciens*

Introduction

Broccoli (*Brassica oleracea* var. *italica*) is one of the most nutritious vegetable crops rich in calcium, antioxidants, vitamin A, vitamin K, β -carotene, riboflavin, and iron (Suri et al. 2005). Genetic improvement programs could be a reliable approach to tackle the cultivation problems of the broccoli plants. Genetic transformation is effective and less time-consuming compared to other methods of genetic improvement including conventional breeding (Cardoza and Stewart 2003; Hong et al. 2009). The presence of an efficient transformation protocol is the basic needs in genetic improvement of a crop via *Agrobacterium*-mediated transformation. The most extensively applied technique for genetic transformation of *Brassica* spp, including cabbage and broccoli, is the *Agrobacterium tumefaciens*-mediated method (Cardoza and Stewart 2003, 2004). *Agrobacterium rhizogenes* has also been applied for transformation of several members of the *Brassica* family (Henzi et al. 2000). However, in *A. rhizogenes*-mediated transformation, production of transgenic plants with abnormal phenotypes after regeneration from the hairy-root cultures is usually observed, which is a disadvantage of this method (Puddephat et al. 2001; Young et al. 2003). The pre-requisite to the realization of such gene transfer method is the availability of an efficient plant regeneration

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system. Several important factors that affect the transformation efficiency need to be optimized for achieving a high frequency of *Agrobacterium*-mediated transformation (Opabode 2006). The factors include pre-treatment of explants (Hiei et al. 2006), bacterial concentration (Rafat et al. 2010) and immersion time (Xing et al. 2007), co-cultivation condition (Vasudevan et al. 2007) as well as bacterial strain, plant genotype and explant type (Chen et al. 2001).

‘Green Marvel’ is one of the commonly grown broccoli cultivars in the cool climate regions of the world. The cultivar, which could also be grown on the highlands of the tropics, responds adversely to extreme temperatures and high humidity in the lowland; thus gene transformation is essential for improving its tolerance against heat stress. Heat shock proteins (HSPs) are functionally related proteins involved in the folding and unfolding of other proteins. Their expression is increased when cells are exposed to elevated temperatures or other stresses (Narberhaus 2010; Omar et al. 2011). HSP101 appears to play a major and specific role in conferring acquired thermotolerance (Wahid et al. 2007; Su and Li 2008). Transgenic broccoli plants have shown improved tolerance to dehydration, as well as to other types of stresses (salt, heavy metals, and hydrogen peroxide) (Vinocur and Altman 2005). The production of heat-tolerant rice via *Agrobacterium*-mediated transformation with *Arabidopsis thaliana* HSP 101 (*AtHSP101*) cDNA (Katiyar-Agarwal et al. 2003) established the crucial role of *AtHSP101* in the heat tolerance characteristic (Hong and Vierling 2000; Queitsch et al. 2000; Rafat et al. 2010).

The main objectives of this study were to establish an efficient regeneration system using shoot tip culture in broccoli cv. Green Marvel followed by the introduction of *AtHSP101* cDNA into the cultivar via *Agrobacterium tumefaciens*-mediated transformation and the optimization of parameters that affect the transformation as the first step to increase the heat tolerance characteristic of the crop.

Materials and methods

Plant material and seed germination

Hybrid seeds of broccoli cv. Green Marvel were bought from Kepong, Kuala Lumpur, Selangor, Malaysia. The seeds were placed under running tap water for 15 min followed by immersion and shaking in 70 % ethanol for 1 min. The seeds were rinsed once with sterile distilled water followed by immersion and shaking in NaOCl (1.05 % sodium hypochlorite) with 1–2 drop(s) Tween 20 for 10 min. Finally, the seeds were rinsed with sterile distilled water three times and cultured on a germination medium consisting of half-strength MS (Murashige and Skoog 1962) salts supplemented with 2.8 g l⁻¹ phytagel and 30 g l⁻¹ sucrose. The sterilization of seeds was carried out according to Ravanfar et al. (2014b) with some modifications.

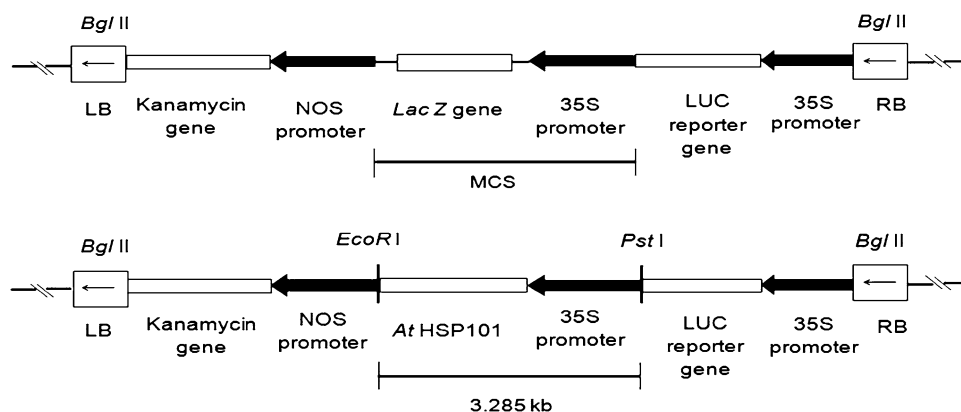
Explant preparation and shoot regeneration

Well-defined shoot tips (8 mm in height) were isolated from 6-day-old seedlings and placed vertically on medium containing different concentrations of zeatin (0, 0.1, 0.5, 1, 1.5, 2 and 3 mg l⁻¹) for shoot regeneration. Each treatment consisted of three replications and each replication per treatment contained 10 explants. Parameters recorded were the percentage of explants producing shoots and mean number of shoots produced per explant at week 8 of culture.

Bacterial strains, binary plasmid vectors, and transformation procedure

The *A. tumefaciens* strain LBA4404 carrying the binary plasmid vector pGreen 0049 was used in the transformation experiments. The pGreen0049 vector contains the antibiotic resistance gene (kanamycin) as the plant selectable marker (Fig. 1). Therefore, the minimum inhibitory

Fig. 1 *AtHSP101* cDNA was cloned into pGreen0049. Expression of *AtHSP101* was driven by the CaMV 35S promoter. Source: <http://www.pgreen.ac.uk/JIT/pG0049.htm>



concentration (MIC) of kanamycin was assessed by culturing shoot tip explants on a shoot regeneration medium consisting of MS salts supplemented with the best zeatin concentration and 0, 10, 20, 30, 40, 60, 70, 80, 90 and 100 mg l⁻¹ kanamycin. In the following experiment, the effect of acetosyringone (AS) (0 and 200 µM) in combination with pre-culture treatments [without pre-culture, 4 days of pre-culture on callus induction medium (CIM) and 4 days of pre-culture on SRM] on efficiency of transformation was assessed by the application of the bacterial dilution of 1:10 and 5 min inoculation time of Rafat et al. (2010). CIM consisted of MS medium supplemented with 1 mg l⁻¹ 6-benzylaminopurine (BAP) and 0.1 mg l⁻¹ naphthalene acetic acid (NAA). The LBA4404 strain harboring the pGreen 0049 vector was grown in Luria–Bertani (LB) broth containing 50 µg l⁻¹ kanamycin and incubated overnight at 28 °C with shaking at 200 rpm until the OD₆₀₀ reached 1.6. AS at 0 and 200 µM was added to the *Agrobacterium* growth medium and the shoot tip explants were given the pre-culture treatments prior to inoculation into the medium. In the next experiment, the effect of *Agrobacterium* (OD₆₀₀ = 1.6) dilution of 1:5, 1:10 and 1:15 and inoculation time of 5, 15 and 30 min were assessed on the efficiency of transformation using the best pre-culture combination obtained earlier. After the infection with *Agrobacterium*, the shoot tip explants were co-cultivated for 4 days in the dark at 25 °C on SRM containing MIC of 80 mg l⁻¹ kanamycin for the selection of putative transformed shoots. The SRM also contained 150 mg l⁻¹ ampicillin to eradicate *Agrobacterium* growth. Non-inoculated shoot tip explants were regenerated on SRM as a control. The parameters recorded were the percentage of regeneration of survived explants (explants that remained green) and mean number of shoots obtained per survived explant. The experiments were arranged in a Completely Randomized Design. Each treatment was replicated three times and each replication per treatment contained 10 explants. Data were analysed using the analysis of variance (ANOVA). Duncan New Multiple Range Test (DNMRT) at $\alpha = 5\%$ was used for comparison between treatment means. Data on the percentage of putative shoot formation in the experiments combining AS with preculture and bacterial dilution with inoculation time were transformed to log₁₀ ($Y + 0.1$) due to high coefficient of variation.

Molecular analyses of transformants

Genomic DNA was extracted from 100 mg fresh leaves of putative transformants on week 14 after co-cultivation and the control plants (regenerated from non-inoculated explants) using DNeasy Plant Mini Kit QIAGEN. Two pairs of primers were designed and used in the polymerase

chain reaction (PCR) to amplify the 500 and 690 bp *AtHSP101* cDNA region from the total DNA isolated from the shoot tip explants. Based on the 500-bp *AtHSP101* sequence, a forward (5' GACATCATGGTGTGCGAATCC 3') and a reverse primer (5' GGCCCAACGTTTTCTGTGAGC 3') and for the 690-bp *AtHSP101* a forward (5' GGCGAGGGTAAAGTCTGAGG 3') and a reverse (5' GATTTCGCACACCATGATGTCC 3') primer were designed. PCR amplification was carried out using Green Taq (2×) protocol (Green PCR Master Mix) in 50 µl reaction volume containing 25 µl Master Mix PCR buffer, 1 µM forward primer (5 µl), 1 µM reverse primer (5 µl), 1 µg genomic DNA (5 µl), and water (nuclease-free) (10 µl). The PCR conditions were set for an initial denaturation step of 3 min at 95 °C and subsequent 36 cycles at 95 °C for 45 s, 53.6 °C for 30 s (for 500 bp *AtHSP101* cDNA) or 56.5 °C for 30 s (for 690 bp *AtHSP101* cDNA), 72 °C for 1 min/kb followed by a final extension step at 72 °C for 7 min. PCR amplified products were analyzed by electrophoresis in 1 % (w/v) agarose–ethidium bromide gel.

For Southern blot analysis, genomic DNA from transgenic broccoli leaf tissues was extracted using cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle 1990) with some modifications. Dig High Prime DNA Labeling and Detection Starter Kit II (Roche) was used for the detection of independent transgenic lines of broccoli cv. Green Marvel. The step for Southern blot analysis consisted of DNA labeling, DNA transfer and fixation, hybridization with a probe and immunological detection. For DNA labeling, DIG-labeled DNA probes were regenerated with DIG-High Prime according to the random primed labeling technique. PCR product amplified with forward primer: 5' GGCGAGGGTAAAGTCTGAGG 3' and reverse primer: 5' GATTTCGCACACCATGATGTCC 3' and extracted from 1 % agarose gel was used to prepare the probes for Southern hybridization. The probes contained a common region to *HSP* gene. For DNA transfer and fixation, 20 µg genomic DNA isolated from fresh leaves of three shoot tip transgenic broccoli lines and the control were digested with *EcoRI* enzyme whose restriction site is absent in the T-DNA. The digested genomic DNAs were fractionated in 1 % agarose gel and then blotted on nylon membrane through capillary transfer by placing the nylon membrane on the gel and incubating in 20× SSC for 17 h. For hybridization of membrane with the probe, the optimum hybridization temperature was 42.31 °C calculated according to the manual structure of Dig High Prime DNA Labeling and Detection Starter Kit II (Roche). Finally, for immunological detection, the membrane was incubated in 20 ml antibody solution containing 150 U/ml Anti-digoxigenin-Ap for binding to the DIG-labeled probe. The membrane was covered with 1 ml disodium (CSPD) (substrate for alkaline phosphatase) and

incubated for 10 min at 37 °C. After 1–5 min at 25 °C the blue bands (chemiluminescence) were captured using a Canon camera in the dark room.

In the luciferase assay, cell lysates were prepared from the control plants and the eight transformants, one at a time, according to the protocol of Ow et al. (1986) with some modifications. The cell lysate (20 µl) was mixed with 100 µl of luciferase assay reagent and the light produced was measured using a luminometer (Luciferase Assay Kit, Instruction Manual). The results were expressed in terms of relative light units (RLU) per microgram of protein. In the RT-PCR assay, total RNA was extracted from 100 mg of fresh leaves of the transgenic lines and control plants using RNeasy Plant Mini Kit QIAGEN according to the manufacturer's specifications. The QIAGEN OneStep RT-PCR Kit was used with specifically designed forward (5' GGC GAGGGTAAAGTCTGAGG 3') and reverse (5' GATTC GCACACCATGATGTCC 3') *AtHSP101* primers to screen the integration of *AtHSP101* cDNA and for detection of the transcripts. Reaction conditions were set up following the Kit's protocol. The RT-PCR amplification products were visualized by electrophoresis in 1 % (w/v) agarose–ethidium bromide gel.

Results and discussion

Shoot tip explants placed on MS medium containing different concentrations of zeatin began to elongate and produced shoots after 4 weeks of culture. At week 8 of culture, significant differences were observed between the treatments on percentage of explant forming shoots (Fig. 2a) and mean number of shoots produced per explant (Fig. 2b). Percentage shoot production at 1.5 mg l⁻¹ zeatin was among the highest (96.67 %), while the lowest percentage (70 %) was with 3 mg l⁻¹ zeatin treatment at week 8 of

culture. The number of shoots formed from the shoot tip explants was the highest (4.27) at 1.5 mg l⁻¹ zeatin but declined significantly at 2 and 3 mg l⁻¹ zeatin. Therefore, zeatin at 1.5 mg l⁻¹ (Fig. 3a, b) was selected for genetic transformation of broccoli cv. Green Marvel using shoot tip explants in subsequent experiments. The study showed that the different concentrations of zeatin affected shoot development and multiplication from shoot tip explants of broccoli cv. Green Marvel; however, zeatin at 1.5 mg l⁻¹ had effectively broken the apical dominance and caused multiple shoot proliferation. It was also observed that callus was not produced even at high concentrations of zeatin while the shoots produced were normal. The superior effect of zeatin on shoot proliferation from shoot tip explants was earlier reported in *Brassica campestris* (Lim et al. 1997). In addition, Munir et al. (2008) reported obtaining the best regeneration when *B. napus* explants were cultured on medium containing 1 mg l⁻¹ zeatin. Other species of *Brassica* also responded well on medium with 1.5 mg l⁻¹ zeatin (Cardoza and Stewart 2006), which indicates that zeatin is generally good for the *Brassica* spp. However, Metz et al. (1995) and Ravanfar et al. (2014a) reported high regeneration of peduncle, hypocotyl and petiole explants on medium containing either BAP or thidiazuron (TDZ).

The MIC determination of kanamycin was carried out for the shoot tip explants of broccoli based on the presence of the plant selectable marker gene in the T-DNA region of pGreen vector. Among the highest percentage of regeneration from the survived shoot tip explants (96.67 %) was in the control which differed significantly compared to other treatments except with 10 and 20 mg l⁻¹ kanamycin (Table 1). However, maximum death (100 %) occurred on 90 and 100 mg l⁻¹ kanamycin (Fig. 3c). Treatments without and with low concentrations of kanamycin (0, 10 and 20 mg l⁻¹) produced many shoots from the surviving shoot

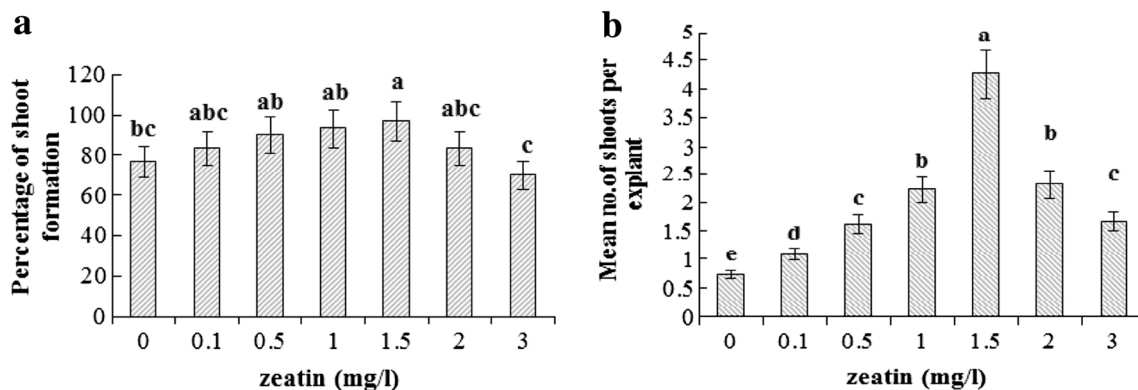


Fig. 2 Effect of different concentrations of zeatin on shoot formation of broccoli cv. Green Marvel at week 8 of culture. **a** Percentage of shoot formation and **b** mean number of shoots produced per shoot tip

explants. Bars followed by the same letter are not significantly different based on DMNRT at $p = 0.05$

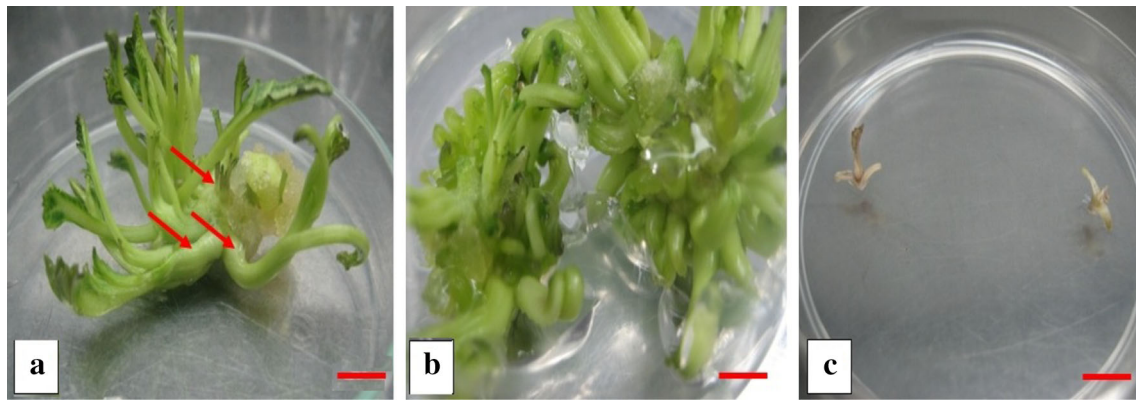


Fig. 3 Shoot multiplication and explant mortality rate from shoot tip explant of broccoli cv. Green Marvel at week 8 of culture. **a, b** On medium with 1.5 mg l^{-1} zeatin without kanamycin and **c** on medium

with 1.5 mg l^{-1} zeatin and 90 mg l^{-1} kanamycin. *Arrows-shoots a Bar 2.5 mm, b, c Bar 5 mm*

Table 1 Percentage and mean number of shoot regeneration from surviving shoot tip explants of broccoli cv. Green Marvel at week 8 of culture on SRM supplemented with different concentrations of kanamycin. Means followed by the same letter are not significantly different based on DMNRT ($p = 0.05$)

Kanamycin (mg l^{-1})	Shoot regeneration (%)	Mean number of shoots per explant
0 (control)	96.67 a	3.93 a
10	93.33 a	1.77 b
20	86.67 ab	1.67 c
30	76.67 b	1 cd
40	53.33 c	0.80 d
60	46.60 c	0.30 e
70	20.00 d	0.17 e
80	3.33 e	0.03 e
90	0.00 e	0.00 e
100	0.00 e	0.00 e

tip explants at week 8 of culture (Table 1). According to Rafat et al. (2010) if any antibiotic resistance gene as a plant selectable marker is available inside the vector construct, the MIC of the antibiotic has to be determined before transformation to allow the selection of putative transformants. Kanamycin has been used as a selectable marker in genetic transformation of several *Brassica* species. Metz et al. (1995) investigated the selection of putative transgenics on medium with 25 mg l^{-1} kanamycin in broccoli peduncle explants. Li et al. (1999) used 15 mg l^{-1} kanamycin to select transformed shoots from cotyledon explants of *B. napus*, while Block et al. (1989) and Cao and Earle (2003) applied 50 mg l^{-1} kanamycin for the selection of putative transformants from *B. oleracea* hypocotyl explants. Meanwhile Zhao et al. (2008) selected putatively transformed shoots of mustard (*B. juncea* Coss.) using 30 mg l^{-1} kanamycin. In this study, the ability of shoot tip explants of

B. oleracea cv. Green Marvel to withstand high concentration of kanamycin (80 mg l^{-1}) could be due to the fact that the cells in the shoot tip are more tolerant to kanamycin because of the continuous cell division process that occurs in that region. Results showed that the highest mortality rate occurred on medium containing 90 mg l^{-1} kanamycin. Therefore, 80 mg l^{-1} kanamycin was selected as the MIC for effective screening of putative transformants from shoot tip explants of broccoli cv. Green Marvel following *Agrobacterium*-mediated transformation.

Optimization of plant transformation conditions

Shoot tips were placed on different combinations of preculture and AS treatments for optimizing the *Agrobacterium tumefaciens*-mediated transformation protocol. Selection of putative transformants from the non-putatives was done by culturing on SRM supplemented with 150 mg l^{-1} ampicillin and 80 mg l^{-1} kanamycin for 8 weeks. Shoot tip explants precultured on CIM and exposed to $200 \mu\text{M}$ AS produced among the highest percentage of putative transformants (23.33 %) (Fig. 4a) as well as among the highest mean number of putative transformants (0.27) at week 8 of culture (Fig. 4b). According to Khan et al. (2009), preculturing, AS treatment and explant source affected transformation frequency. In this study, the highest putative transformant production occurred with preculture and with AS application for shoot tips of broccoli cv. Green Marvel. Preculture and AS application were also required for efficient transformation in other *Brassica* spp. (Chakrabarty et al. 2002; Rafat et al. 2010; Kuta and Tripathi 2005). In broccoli cv. Green Marvel, preculture of shoot tip explants on CIM as determined in this study was necessary to overcome necrosis and increased the efficiency of transformation.

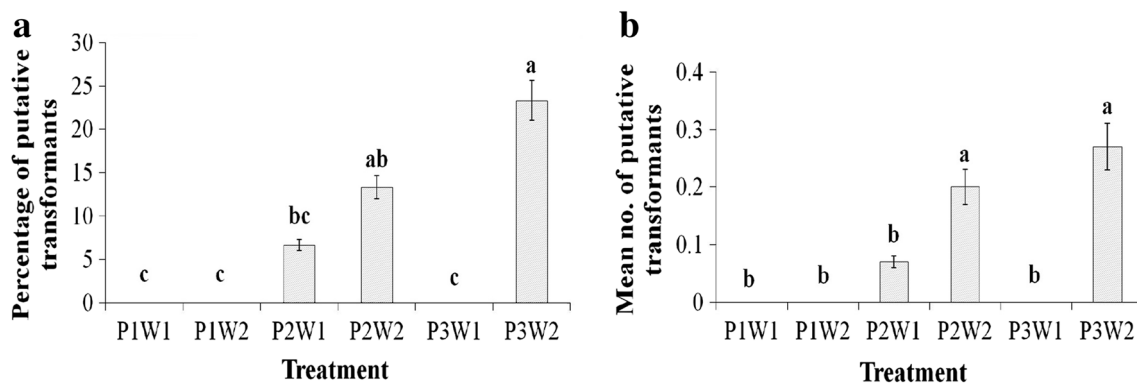


Fig. 4 Effect of preculture and acetosyringone on **a** percentage of shoot formation and **b** mean number of putative transformants produced per shoot tip explant of broccoli cv. Green Marvel at week 8 of culture on SRM with 150 mg l^{-1} ampicillin and 80 mg l^{-1} kanamycin. Bars followed by the same letter are not significantly different from each other based on DMNRT ($p = 0.05$). *P1W1*

without preculture without acetosyringone, *P1W2* without preculture with $200 \mu\text{M}$ acetosyringone, *P2W1* precultured on SRM without acetosyringone, *P2W2* precultured on SRM with $200 \mu\text{M}$ acetosyringone, *P3W1* precultured on CIM without acetosyringone, *P3W2* precultured on CIM with $200 \mu\text{M}$ acetosyringone

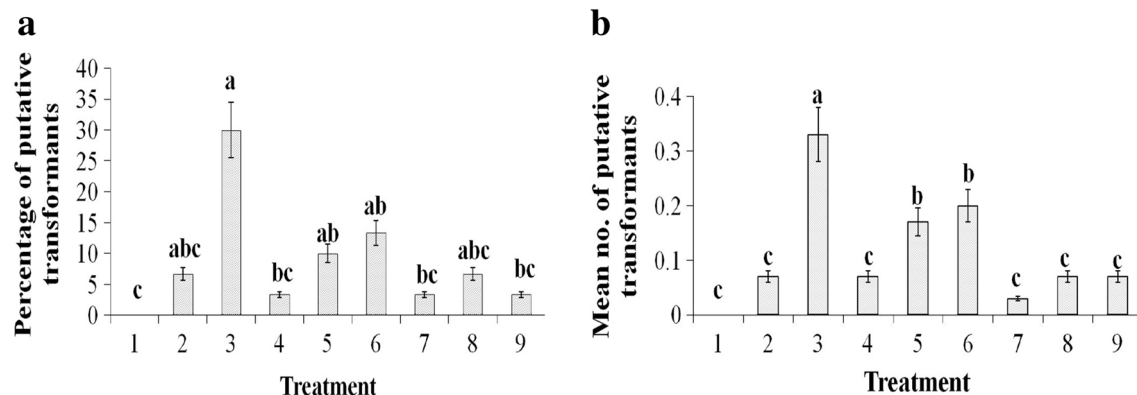


Fig. 5 Effect of bacterial dilution and inoculation time on **a** percentage of shoot formation and **b** mean number of putative transformants produced per shoot tip explant of broccoli cv. Green Marvel at week 8 of culture on SRM with 150 mg l^{-1} ampicillin and 80 mg l^{-1} kanamycin. Bars followed by the same letter are not significantly different based on DMNRT ($p = 0.05$). Treatments: 1:1:5 bacterial

dilution, 5 min inoculation, 2:1:5 bacterial dilution, 15 min inoculation, 3:1:5 bacterial dilution, 30 min inoculation, 4:1:10 bacterial dilution, 5 min inoculation, 5:1:10 bacterial dilution, 15 min inoculation, 6:1:10 bacterial dilution, 30 min inoculation, treatment 7:1:15 bacterial dilution, 5 min inoculation, 8:1:15 bacterial dilution, 15 min inoculation, 9:1:15 bacterial dilution, 30 min inoculation

Figure 5a, b, shows that shoot tip explants exposed to a high concentration of *Agrobacterium* (1:5 dilution) and relatively long period of inoculation (30 min) (Treatment 3) exhibited the highest transformation efficiency. However, Chakrabarty et al. (2002) reported that using 1:10 dilution of *A. tumefaciens* and 10 min inoculation time resulted in a relatively high percentage (7.6 %) of recovery of transformants. In general, the results in this study confirmed earlier observations that the appropriate bacterial density and inoculation time help to improve transformation frequency of *Brassica* (Yi et al. 2011). After selecting the putative broccoli transformants, hormone-free MS medium (Ravanfar et al. 2009) without kanamycin was used for elongation and rooting of the transformed shoots for further molecular analysis (Fig. 6).

Molecular analyses of *AtHSP101* transgenics

The expected amplified DNA fragments from the PCR were 500 and 690 bp for *AtHSP101*. The resulting samples were subjected to electrophoresis using a 1.0 % agarose gel. Electrophoresis showed that the product sizes were that of the expected amplification sizes. The PCR results indicated stable transformation of the shoot tip explants. Three independent lines showed the expected fragments of 500 and 690 bp for *AtHSP101* in the PCR products (Fig. 7a, b). The PCR results also showed a transformation efficiency of 8.33 %. Kong et al. (2009) reported reaching a maximum transformation efficiency of 18.93 % (positive PCR) when cotyledonary nodes of *Brassica napus* were infected for 10 min with an

Fig. 6 Acclimatization of broccoli transformants. **a** Rooted transgenic broccoli cv. Green Marvel in a culture flask, **b** after 1 month in plastic pots, **c** after 3 months and **d** after 4 months acclimatization at 34 °C in the transgenic greenhouse (Ravanfar et al. 2013) Acclimatization of broccoli transformants. **a**, **b**, **d** Bar 30 mm and **c** Bar 50 mm

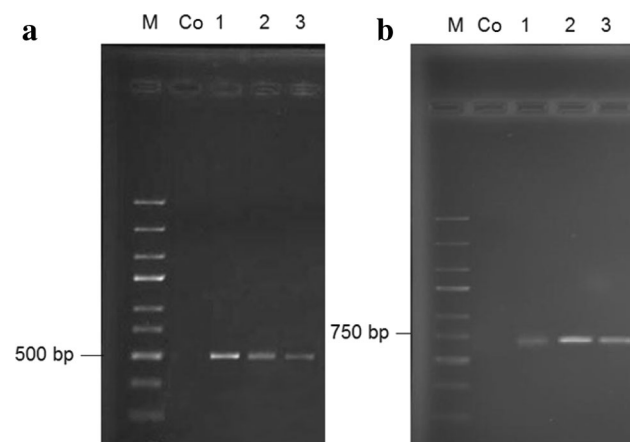
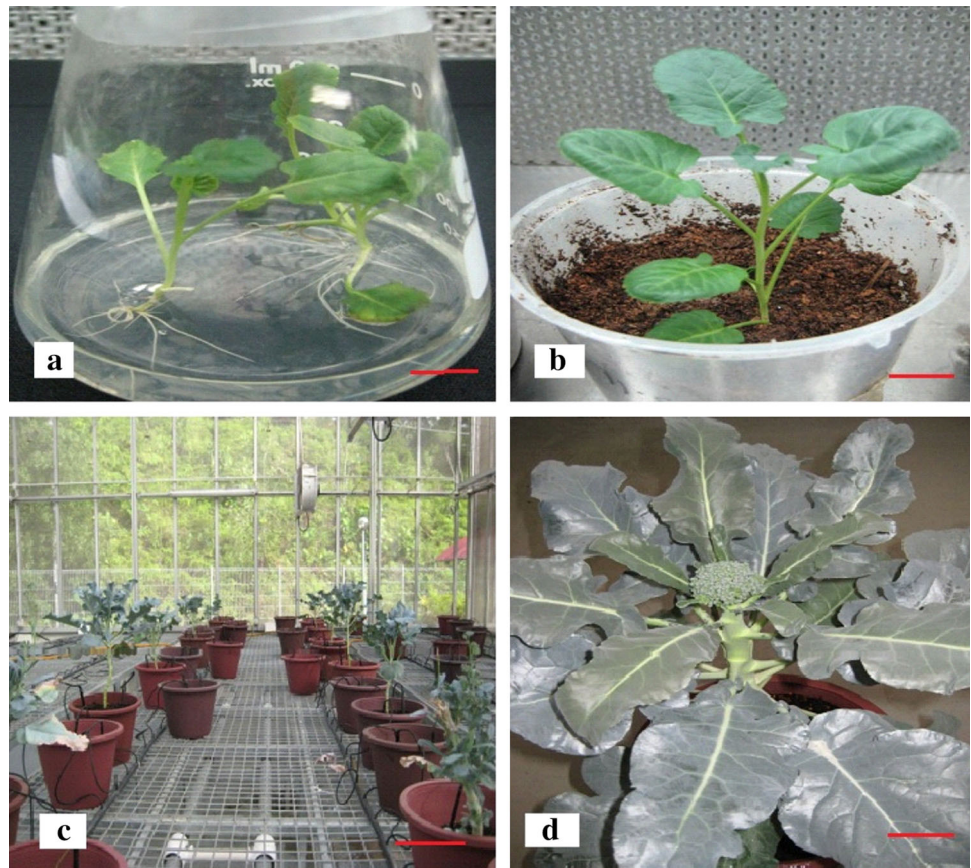
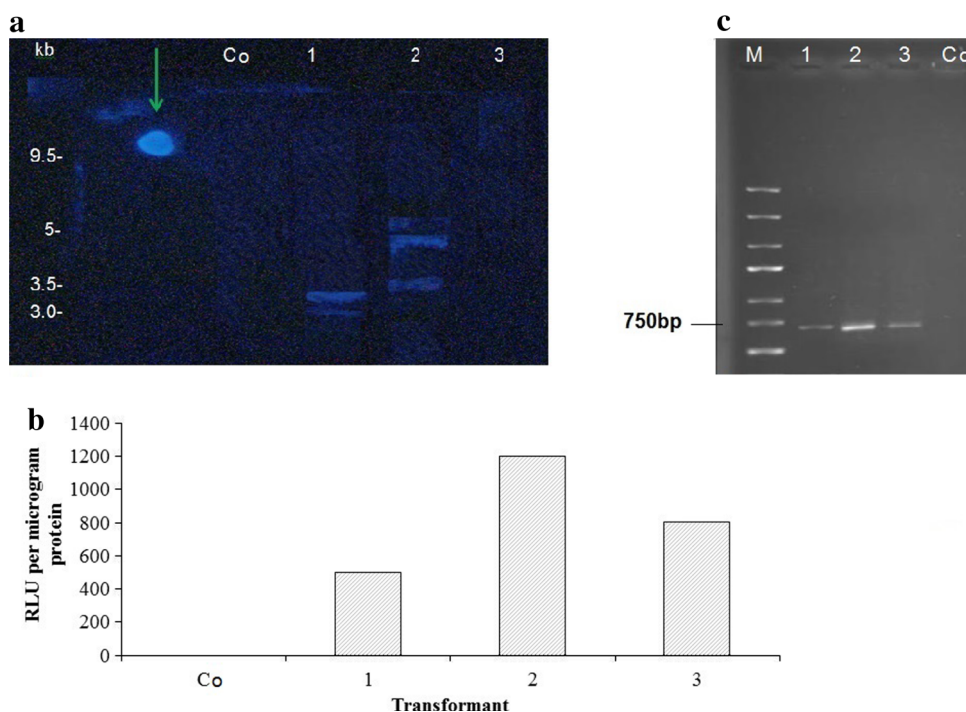


Fig. 7 PCR analysis of DNA from putatively transformed shoot tip-derived regenerants of broccoli cv. Green Marvel using primers of *AtHSP101* cDNA. **a** 500 bp fragments were amplified by the first set of primers and **b** 690 bp fragments were amplified by the second set of primers. *M* marker (0333), *Co* non-transformed plant (control), lanes 1–3 transformed regenerants

Agrobacterium suspension of 0.8 OD and containing 200 μ M AS. According to Metz et al. (1995), the overall transformation efficiency for the flowering stalk of broccoli was 6.4 %, while for seedling explants it was 6.6,

10 % for hypocotyl explants and only 1.8 % for petiolar explants. For Southern blot analysis, genomic DNA prepared from the transgenic plants and the control was digested with *EcoR*I, as there is a unique site for the restriction enzyme in the T-DNA region of the binary vectors used. The size of the expected hybridization band was slightly more than 3 kb, due to the size of *AtHSP101*. Figure 8a shows two different hybridization bands of the *AtHSP101* in the shoot tip derived transformants. Meanwhile, from three independent transgenic lines assessed only two lines showed positive hybridization bands carrying two and three copies of the gene. No positive hybridization bands were found in the untransformed plants (control) and line 3. Thus, the Southern blot analysis confirmed that the *AtHSP101* gene had been stably integrated into the genome of two transgenic lines. Stable integration of transgene into the genome of other *Brassica* species has been demonstrated by Southern blot analysis such as in *B. oleracea* var. *botrytis* (cauliflower) (Nugent et al. 2006), *B. oleracea* (Chinese cabbage) (Min et al. 2007) and *B. napus* (rapeseed) (Cheng et al. 2010). Transgenic plants confirmed by PCR and Southern blot analysis were further analyzed using luciferase assay and RT-PCR for the presence of *AtHSP101* transcripts.

Fig. 8 Molecular characterization of independent PCR-positive lines of broccoli cv. Green Marvel. **a** Southern blot analysis, *arrow* indicates probe of binary vector pGreen 0049 (positive control), Co—non-transformed plant (negative control), lanes 1, 2 and 3 shoot tip-derived transformants. **b** Luciferase activity in RLU per microgram protein of shoot tip-derived transformants. Co—control, 1–3 shoot tip derived transformants. **c** RT-PCR analysis of transgenic lines derived from shoot tip explant. M marker, lanes 1–3 transformed lines from shoot tips, Co control



According to Chen et al. (2001) RT-PCR revealed that *ipt* gene expression occurred early on the day of detachment of broccoli and Southern blot showed the hybridization analysis of *ipt* transformants. The *luciferase* gene has been used extensively as a reporter gene in the verification of transgenic tobacco (Ow et al. 1986) and rice (Sadasivam and Gallie 1994, Cornejo et al. 1993). The results from the luciferase assays indicated that the *luciferase* gene could be suitably used as a reporter gene for the confirmation of transgenic plants. The activity of the *luciferase* gene was visualized in the shoot tip-derived broccoli transformants (Fig. 8b). The highest RLU per microgram protein was in shoot tip transformants 3 (1200 RLU) followed by transformant 4 (800) and 2 (500). Non-transformed shoot tip-derived regenerants as controls did not show any RLU value. The transgenic status of the three independent transgenic lines that exhibited luciferase activity was further assessed by RT-PCR. All three transgenic lines showed the expected 690 bp amplified fragment in RT-PCR (Fig. 8c) demonstrating transcriptionally active *AtHSP101* gene in the plant genome. The introduction of the *AtHSP101* gene into broccoli cv. Green Marvel is the first step towards the production of broccoli plants tolerant to high temperature via genetic transformation.

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