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Constitutive and salt-inducible expression of *SlBADH* gene in transgenic tomato (*Solanum lycopersicum* L. cv. Micro-Tom) enhances salt tolerance

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ABSTRACT

To improve the stress tolerance of crops, many genes, including transcription factors, have been expressed in transgenic plants using either constitutive or stress-inducible promoters. However, transgenic plants that show strong constitutive expression of transcription factors often suffer from many undesirable phenotypes, such as stunted growth and reduced yield. In the present study, the betaine aldehyde dehydrogenase (BADH) gene, cloned from *Suaeda liaotungensis* and, controlled by the *Cauliflower mosaic virus* (CaMV) 35S promoter or stress-inducible promoter of BADH (P5: −300 to +62 bp), was transformed into tomato (*Solanum lycopersicum*). The transformants with single copy of *SlBADH* were determined by real time PCR. Expression of *SlBADH* in the P5:BADH transgenic plants exhibited salt induced and was higher than that in CaMV35S:BADH under salt stress. The *SlBADH* enhanced salt tolerance of P5:BADH and CaMV35S:BADH transformants. And *SlBADH* in P5:BADH plants did not affect the growth of transformants. Consequently, we conclude that the P5 promoter can drive increased expression of *SlBADH* in transgenic tomato under salt stress and increase salt tolerance without affecting plant growth.

1. Introduction

Drought, salt loading, and freezing are stresses that cause adverse effects on plant growth and crop yield. Plants utilize a number of protective mechanisms to maintain normal cellular metabolism and prevent damage to cellular components. One common metabolic adaptation to salinity stress is the accumulation of osmoprotectants. One of these osmoprotectants, glycine betaine (GB), is a bipolar quaternary ammonium compound accumulated in many plant species [1]. It provides tolerance to osmotic stress by stabilizing the structure of proteins and adjusting the osmotic potential in the cytoplasm to maintain the water status [2]. Synthesis of GB is catalyzed by betaine aldehyde dehydrogenase (BADH), which is encoded by the *BADH* gene and converts betaine aldehyde into GB in some halotolerant plants.

The *BADH* gene has been cloned from many plant species, such as *Spinacia oleracea* [3], *Hordeum vulgare* [4], and *Suaeda liaotungensis* [5]. In spinach, the specific activity of BADH rose 3-fold when plants were grown in the presence of 300 mM NaCl [6], and the levels of BADH protein and *BADH* mRNA increased several-fold at concentrations between 0 and 500 mM NaCl [7,8]. The *BADH* mRNA level increased almost 8-fold in barley leaves when plants were treated with 300 mM NaCl, and decreased upon release from the stress, whereas the level did not decrease under continuous salt stress [4]. These observations indicated that expression of the *BADH* gene was induced by salinity.

The promoter is an important *cis*-regulatory element for gene expression and plays a critical role in plant gene expression and regulation. Constitutive promoters are used to drive alien gene expression in most transgenic engineering procedures. For example, expression of 35S::ZmCBL4 complemented the salt hypersensitivity and enhanced salt tolerance in *Arabidopsis thaliana* [9]. The constitutive promoter OsCc1 was effective in expressing the gene *AP37*, which encodes an AP2 domain transcription factor that confers tolerance to drought stress in rice [10]. Transgenic 35S::JcDREB Arabidopsis plants were tolerant to freezing and salt stress [11].

Although constitutive promoters can improve the abiotic stress tolerance of transgenic plants, expression of the alien gene may cause stunted growth and reduction of yield. For example, stress-tolerant transgenic Arabidopsis expressing 35S:DREB1A displayed growth retardation and severe reduction in seed production [12,13]. Growth retardation and low reproductive yields were observed in stress-tolerant transgenic rice that expressed Ubi1:OsNAC6 [14]. Morphological observations showed that 35S:DREB1A transgenic peanuts exhibited stunted growth under control conditions [15]. Therefore, inducible promoters, which can drive expression of the alien gene only during exposure to stress, are of importance for genetic engineering. Kasuga [13] used the

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stress-inducible rd29A promoter to drive expression of *DREB1A* in Arabidopsis, and observed improved stress tolerance of the transgenic plants and much improved growth under non-stress conditions. Peanut transformants carrying rd29A:DREB1A did not show any growth retardation either *in vitro* or in the greenhouse [15]. Transgenic mulberry plants carrying the stress-inducible rd29A promoter were able to tolerate salt and drought stress more efficiently than transformants carrying the constitutive CaMV35S promoter [16].

Li et al. isolated the BADH promoter (1993 bp) from *S. liaotungensis*. This promoter contains stress-induced elements [17]. Function analysis of the BADH promoter showed that the smallest promoter fragment, P5 (−300 to +62 bp), is sufficient for NaCl induction. GUS enzyme activity was enhanced 6.3-fold in transgenic tobacco leaves containing the P5 promoter in the presence of 400 mM NaCl compared to non-inductive leaves [18].

In the present study, we transformed the *SlBADH* gene cloned from *S. liaotungensis* [5], controlled by either the CaMV35S promoter or the P5 promoter, into tomato (*Solanum lycopersicum* cv. Micro-Tom) using a leaf-disc transformation system. Molecular analysis of the transgenic plants was performed to screen the transformants and determine the number of *SlBADH* copies in the transformants. The expression of *SlBADH* in P5:BADH and CaMV35S:BADH transgenic plants was detected by real-time PCR. Our objective was to assess the efficiency of the stress-inducible P5 promoter to drive expression of *SlBADH* in Micro-Tom tomato, with the aim of enhancing tolerance to salt stress and minimizing the negative effects on growth in transgenic plants compared with the use of the CaMV35S promoter.

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds of *S. lycopersicum* cv. Micro-Tom (PanAmerican Seed Co., Chicago, IL) were surface sterilized and placed on 1/2 Murashige and Skoog (MS) solid medium, and incubated at 25 °C under a 16 h photoperiod (about 3000 lux provided by cool white fluorescent lamps) for about 7 days until the cotyledons had expanded fully and the true leaves were several millimeters long.

2.2. Development of transgenic Micro-Tom plants

Transformation was performed using *Agrobacterium tumefaciens* leaf disc method in accordance with Sun et al. [19]. The *A. tumefaciens* strain LBA4404 with the plasmid pCAMBIA1301-P5-BADH or pCAMBIA1301-CaMV35S-BADH (Fig. 1) were used. Through callus induction, shoot induction, and root induction, hygromycine-resistant transgenic plants were obtained.

2.3. Molecular analysis of transgenic plants

Genomic DNA was extracted from the putative transgenic and non-transgenic (NT) Micro-Tom plants using CTAB protocol [20]. Plasmid pCAMBIA1301-P5-BADH DNA was isolated using the SDS-alkaline denaturation method. The quality of genomic DNA and plasmid DNA was checked with a ultramicro-spectrophotometer (Thermo Nanodrop 2000, Wilmington, DE, USA).

The transgenic plants were identified by Genomic PCR. The gene-specific primers SlBADH-F and SlBADH-R (Table 1) were designed within the region of the known *SlBADH* cDNA sequence [5]. Using this pair of primers, PCR amplification was performed to screen the transgenic plants. NT plants and the plasmid pCAMBIA1301-P5-BADH were used as negative and positive controls, respectively.

For RT-PCR analysis, total RNA was isolated from the PCR positive transformants and NT plants using RNAiso plus (TaKaRa, Dalian, China). Using a Prime Script® 1st Strand cDNA Synthesis Kit (TaKaRa), the first strand cDNA was synthesized. Gene-specific primers SlBADH-F and SlBADH-R were used for *SlBADH* amplification.

The copy number of *SlBADH* in transgenic plants was determined by real-time PCR. The single-copy gene *chromoplast-specific lycopene beta-cyclase* (*B* gene; GenBank No. AF254793) in tomato [21] was used as an endogenous reference gene. The oligonucleotide primers, B-qF and B-qR, and SlBADH-qF and SlBADH-qR (Table 1), were designed with Primer Premier 5.0 software and synthesized by Takara. Standard curves of the *B* and *SlBADH* genes were constructed using serial dilutions of NT Micro-Tom genomic DNA and the plasmid pCAMBIA1301-P5-BADH DNA, respectively. The copy number for each of the *B* and *SlBADH* genes in the initial template was obtained from the standard curves. The copy number of the *SlBADH* gene in transgenic Micro-Tom was obtained by comparing the number of *SlBADH* with *B* in the initial template. All PCRs were performed in triplicate in a total reaction volume of 50 μl with SYBR® Premix Ex *Taq*™ (Perfect Real Time; Takara) in a Takara PCR Thermal Cycler Dice Real Time system TP800 (Shiga, Japan). For each DNA sample, both the target and reference genes were amplified independently on the same plate and in the same run.

2.4. Expression of SlBADH in transgenic plants

The *SlBADH* expression in transgenic plants was detected by real-time PCR. Transgenic plants carrying a single copy of the *SlBADH* gene and NT plants were transferred to 1/10 MS liquid medium. After 7 days, the NT, CaMV35S:BADH and P5:BADH plants were treated with 0, 100 or 200 mM NaCl for 24 h, then total RNA was extracted from leaves using RNAiso Plus (Takara). For cDNA synthesis, 500 ng total RNA was reverse-transcribed with the Takara PrimeScript™ RT Master Mix (Perfect Real Time). The *β-actin* gene (GenBank No. FJ532351.1) of tomato was used as an internal control. To amplify the gene the primers Actin-qF and Actin-qR (Table 1) were designed from the selected unique regions with Primer Premier 5.0 software. SlBADH-qF and SlBADH-qR were used as primers for *SlBADH* amplification. The real-time PCR was performed as described above.

2.5. Salt tolerance of transgenic plants

The salt tolerance of NT and transgenic Micro-Tom plants was evaluated under salt stress conditions. The plants were transplanted to 1/10 MS liquid medium, and cultured in a greenhouse maintained at 25 °C under a 16/8-h light/dark photoperiod at a relative humidity of 60–70%. After 7 days, the plants were transferred to 1/10 MS liquid medium containing 200 mM NaCl. After one week, the growth performance of the plants under the salt stress conditions was observed and photographs recorded.

2.6. Growth of transgenic plants

Transgenic Micro-Tom plants were cultured in MS solid medium, in 1/10 MS liquid medium and in soil. All plants were incubated at 25–28 °C under continuous lighting (about 3000 lux provided by white cool fluorescent lamps). The growth of the P5:BADH and CaMV35S:BADH plants was observed and recorded.

3. Results

3.1. Production of transgenic Micro-Tom plants

Four to six weeks after co-culture, callus started to develop from the co-cultivated explants and adventitious buds began to

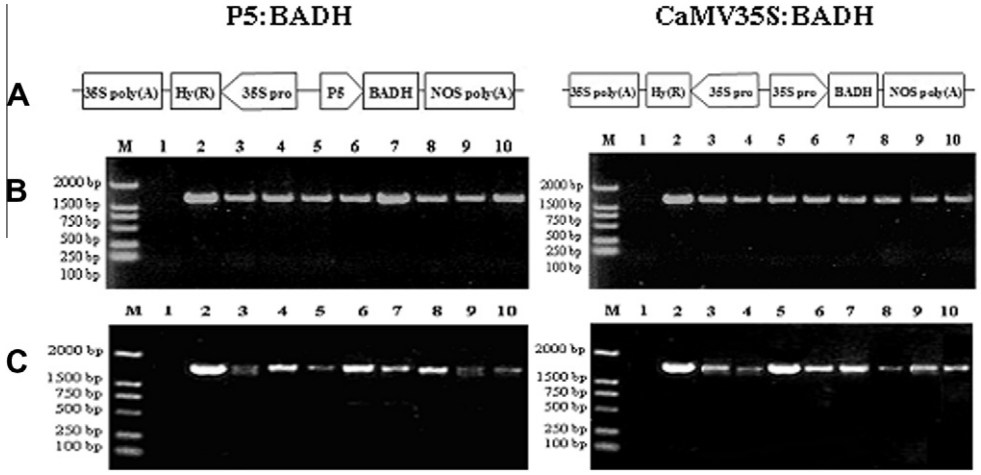

Fig. 1. PCR and RT-PCR analysis of transgenic tomatos expressing *SlBADH*. (A) Structure of the plant expression vectors, pCAMBIA-1301-P5-BADH and pCAMBIA-1301-CaMV35S-BADH; (B) Positive transgenic tomato plants screened by genomic PCR; (C) Positive transgenic tomato plants detected by RT-PCR. M; Dl2000 Marker, Lane 1: Non transgenic tomato; Lane 2:pCAMBIA-1301-P5-BADH or pCAMBIA-1301-CaMV35S-BADH plasmid, Lanes 3–10: transgenic tomato plants.

Table 1
List of primers used in this study.

Primer	Primer sequence
SlBADH-F	5′-ggccatggcgatccctataccttcttcgtc-3′
SlBADH-R	5′-catggtcaccttaaggagacttgtaccacc-3′
SlBADH-qF	5′-tcaccttggaacttggaggtaaa-3′
SlBADH-qR	5′-gtccagaaaacaccaaagatagtcc-3′
B-qF	5′-ccagtactagctgaagccatcgt-3′
B-qR	5′-gccacaaaccattccaaactc-3′
Actin-qF	5′-tccgcgacatgaaggaaaag-3′
Actin-qR	5′-aacggaacctctcagcacca-3′

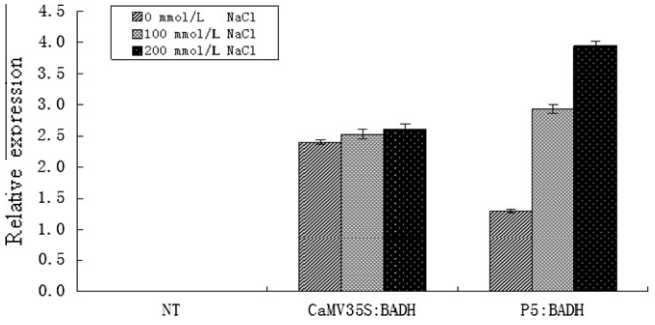

Fig. 2. Relative expression of *SlBADH* in transgenic tomatos regulated by different promoters under different levels of salinity. Plants were treated with 0, 100 or 200 mM NaCl for 24 h. *SlBADH* expression was not detected in non-transgenic (NT) plants grown under both non-stress and salt stress conditions. Higher expression of *SlBADH* was observed in CaMV35S:BADH plants under the non-stress condition and only slightly increased expression under salt stress was detected. In P5:BADH plants, expression of *SlBADH* under the non-stress condition was lower than in CaMV35S:BADH plants and increased markedly under salt stress.

develop on the selection medium, followed by shoot elongation. Shoots were excised from the calli when 3–4 cm long and transferred to rooting medium. Transgenic shoots produced roots within 1–2 weeks after transferring to the rooting medium, and gave rise to Hygromycine-resistant CaMV35S:BADH and P5:BADH transgenic Micro-Tom plants. Positive transgenic tomato plants were detected by genomic PCR and RT-PCR (Fig. 1).

3.2. Number of SlBADH copies in transgenic plants

The positive P5:BADH and CaMV35S:BADH transgenic plants screened by genomic PCR and RT-PCT were used for *SlBADH* copies analysis. Using real-time PCR, we constructed standard curves for the *SlBADH* and *B* genes. After obtaining the C_t values for the *SlBADH* and *B* genes in each plant, we calculated the *SlBADH* and *B* gene copy number in the initial template from the standard curves. The number of *SlBADH* gene copies in the transgenic plants was calculated with the formula 2× BADH copies/B copies. Three lines of P5:BADH transgenic plants and three lines of CaMV35S:BADH transgenic plants contained a single copy of *SlBADH*.

3.3. Expression of SlBADH in transgenic plants

The expression of *SlBADH* in transgenic plants that carried a single copy of the insert under 0, 100 or 200 mM NaCl stress was detected by real-time PCR. No. *SlBADH* expression was detected in NT plants either under no stress or salt stress. Higher expression of *SlBADH* in CaMV35S:BADH plants was observed under the non-stress condition and only a slight increase in expression was detected in response to salt stress. In P5:BADH plants, expression of *SlBADH* was lower under the non-stress condition and increased significantly under salt stress. Hence, *SlBADH* driven by the P5 promoter showed salt-inducible expression in transgenic Micro-Tom. The *SlBADH* mRNA level iP5:BADH plants increased 2.3-fold when exposed to 100 mM NaCl and 3-fold in response to 200 mM NaCl. Under salt stress, the expression of *SlBADH* in P5:BADH plants was much higher than that in CaMV35S:BADH plants (Fig. 2).

3.4. Salt tolerance of transgenic plants

Leaf necrosis and senescence were more prominent in NT plants than in CaMV35S:BADH and P5:BADH transgenic plants in response to 200 mM NaCl treatment. After exposure for 7 days, the NT plants had wilted, whereas the transgenic plants were still healthy and turgid. Moreover, the P5:BADH transgenic plants showed increased survival compared to CaMV35S:BADH plants under salt stress (Fig. 3).

3.5. Growth of transgenic plants

Transgenic Micro-Tom plants were cultured in MS solid medium, in 1/10 MS liquid medium, and in soil. Morphological observations showed that CaMV35S:BADH plants exhibited stunted growth, whereas P5:BADH plants did not show any growth retardation under all culture conditions (Fig. 4).

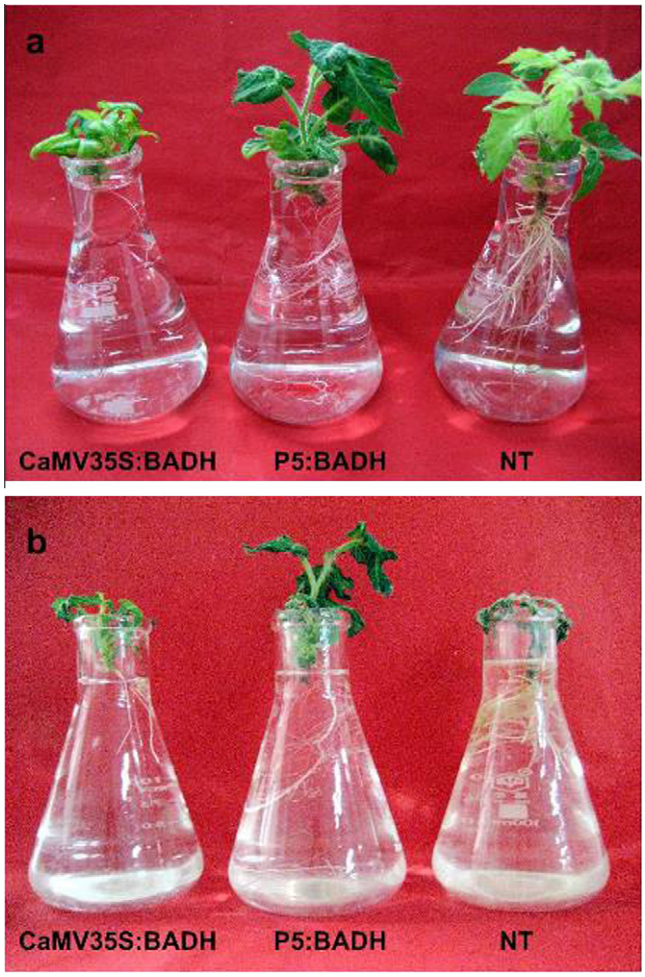

Fig. 3. Salt tolerance of non-transgenic and transgenic tomato Micro-Tom plants. (A) Under the non-stress condition, non-transgenic (NT) plants and CaMV35S:BADH and P5:BADH transgenic plants showed healthy growth. (B) After 7 days of salt stress by treatment with 200 mM NaCl, NT plants had wilted, whereas transgenic plants were healthy and turgid.

4. Discussion

In nature, when exposed to stress conditions, often plants can develop tolerance but this is usually a slow process. To overcome this limitation, many genes involved in a variety of stress responses have been transferred to diverse plants to improve stress tolerance [22–24]. In the present study, we transformed the *SlBADH* gene into Micro-Tom tomato to improve the cultivar's salt tolerance using a constitutive promoter, CaMV35S, and a salt-inducible promoter, P5.

In transgenic plants, the transgene copy number can greatly influence the expression level and genetic stability of the target gene. To some extent, high-copy introgression causes gene silencing, as occurs in diploid plants such as *A. thaliana* [25]. Given that plants with one or two integration events commonly show high-level expression of the exogenous gene [26,27], estimation of the transgene copy number is vital for the selection and cultivation of genetically modified plants. Traditionally, Southern blot analysis is used to estimate the transgene copy number, but this method is laborious, time-consuming, and requires a large quantity of DNA. Real-time PCR provides a fast, inexpensive and high-throughput alternative method [28–30]. In the present study, we estimated the copy number of *SlBADH* in transgenic plants by real-time PCR, and obtained three P5:BADH transgenic Micro-Tom lines and three CaMV35S:BADH transgenic Micro-Tom lines that contained a single copy of *SlBADH*. These transgenic Micro-Tom lines can be used for expression analysis of the exogenous *SlBADH* gene and cultivation of genetically modified Micro-Tom.

To improve stress tolerance, many genes have been transformed into transgenic plants using either a constitutive or stress-inducible promoter. The constitutive promoter CaMV35S is a highly efficient promoter, and desirable genes driven by the CaMV35S promoter have been transferred into many plant species. *SlBADH* was transformed into Micro-Tom using CaMV35S and a salt-inducible promoter, P5, respectively. Real-time PCR analysis detected higher expression of *SlBADH* in CaMV35S:BADH plants under the non-stress condition and slightly increasing under salt stress. In contrast, expression of *SlBADH* in P5:BADH plants under the non-stress condition was lower than that in CaMV35S:BADH plants and significantly increased under salt stress. The *SlBADH* mRNA level in P5:BADH plants increased 2.3-fold when exposed to 100 mM NaCl and 3-fold in response to 200 mM NaCl treatment. Hence expression of *SlBADH* in P5:BADH plants was much higher than that in CaMV35S:BADH plants under salt stress (Fig. 2). This result agrees with the finding that GUS activity driven by P5 promoter was enhanced by 6.3-fold in transgenic tobacco in response to 400 mM NaCl [19]. Similarly, the inducible promoter rd29A is able to direct *SlBADH* expression in Chinese sweetgum plants exposed to salt, cold and drought stress, but with the advantage that expression was absent or undetectable in the non-stress condition [31]. In addition, *osmotin*-transgenic mulberry driven by the rd29A promoter accumulated higher amounts of *osmotin* transcripts than the non-stressed plants, whereas transgenic plants with the CaMV35S promoter showed no significant difference in expression [16].

After treatment with 200 mM NaCl for 7 days, the NT Micro-Tom plants had wilted, whereas the transgenic plants remained healthy and turgid, and the P5:BADH transgenic plants showed better survival than the CaMV35S:BADH plants. Thus we concluded that P5:BADH transgenic Micro-Tom plants were able to resist salt stress more efficiently than CaMV35S:BADH transgenic plants. Previously, three expression constructs that consisted of the full-length cDNA driven by the drought-inducible promoter of OsLEA3–1 (OsLEA3-H), the CaMV 35S promoter (OsLEA3-S), and the rice Actin1 promoter (OsLEA3-A) were transformed into rice. The transgenic plants showed higher drought tolerance than the NT plants, and the plants in which OsLEA3–1 was regulated by the OsLEA3–1 promoter were more able to resist drought stress than plants driven by the CaMV35S promoter [32]. Transgenic mulberry plants carrying the rd29A stress-inducible promoter were able to tolerate salt and drought stress more efficiently than those plants carrying the CaMV35S constitutive promoter [16]. Thus, a stress-inducible promoter is more efficient than the CaMV35S promoter to achieve increased stress tolerance in transgenic plants.

In some instances, constitutive over expression of exogenous genes hampers the growth of plants and reduces their productivity [13,33]. We observed that CaMV35S:BADH plants exhibited stunted growth, whereas P5:BADH plants exhibited normal growth. Such growth retardation was also reported in CaMV35S:CBF1 (DREB1B) tomato [34,35], whereas in tomato plants in which CBF1 was regulated by a stress-inducible promoter, ABRC1, the plants maintained normal growth similar to the untransformed plants under normal growing conditions [36]. The growth retardation observed in transgenic Arabidopsis plants that expressed 35S:DREB1A was diminished when the stress-inducible promoter rd29A was used in place of CaMV35S [13]. Morphological observations showed that CaMV35S:DREB1A peanut plants exhibited stunted growth under control conditions, whereas rd29A:DREB1A transgenic peanut plants showed no growth retardation either *in vitro* or in a greenhouse [15]. Transformants in which transgene

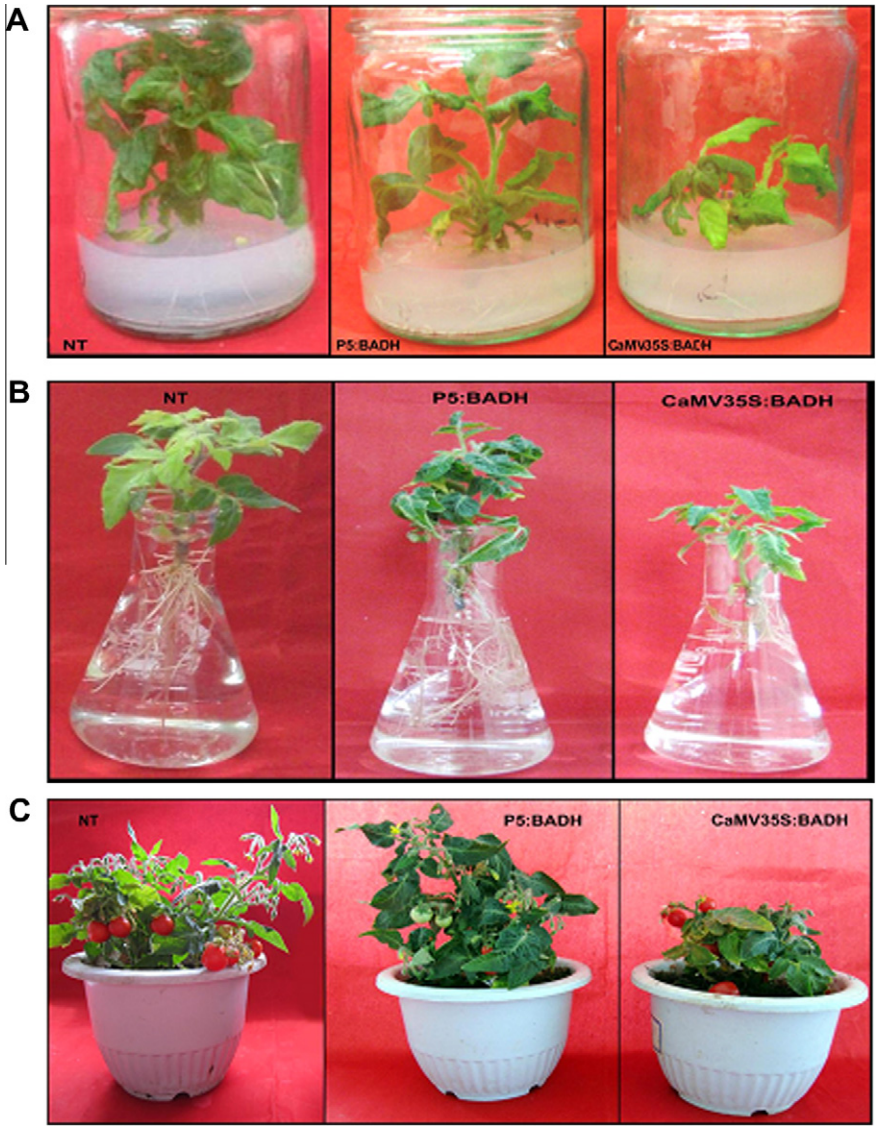

Fig. 4. Growth of NT plants, P5:BADH and CaMV35S:BADH transgenic tomato Micro-Tom plants in growing medium (A), in 1/10 MS liquid medium (B), and in soil (C). Compared to the NT plants, CaMV35S:BADH plants showed highly stunted growth, whereas P5:BADH plants grew normally.

expression is regulated by the constitutive promoter CaMV35S show improved stress tolerance at the expense of plant growth, which is understandable as transgene overexpression can compete for energy and building blocks for the synthesis of protein or RNA that are also required for plant growth under normal conditions. The use of a stress-inducible promoter might overcome this limitation and thereby contribute to normal growth in transgenic plants.

In conclusion, we successfully transformed *SlBADH* into Micro-Tom tomato under the control of the P5 and CaMV35S promoters. Expression of *SlBADH* in CaMV35S:BADH plants was constitutive, and that in P5:BADH plants was salt induced. The *SlBADH* gene driven by the CaMV35S or P5 promoter enhanced salt tolerance of transgenic Micro-Tom plants. And *SlBADH* in P5:BADH plants did not affect the growth of transformants. Our future research will determine the effects of introduction of the *SlBADH* gene controled by the P5 promoter on other physiological traits linked to salt tolerance, agronomic characteristics and yield. We expect that the salt-inducible P5 promoter is widely applicable in plant genetic engineering and may successfully contribute to increased agricultural productivity.

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