Biochemical and Biophysical Research Communications 413 (2011) 10–16

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Overexpression of *PtADC* confers enhanced dehydration and drought tolerance in transgenic tobacco and tomato: Effect on ROS elimination

Bao-Quan Wang [1], Qing-Fu Zhang [1], Ji-Hong Liu *, Guo-Huai Li

Key Laboratory of Horticultural Plant Biology (MOE), College of Horticulture and Forestry Sciences, Huazhong Agricultural University, Wuhan 430070, China

ARTICLE INFO

Article history:
Received 31 July 2011
Available online 19 August 2011

Keywords:
PtADC
Polyamine
Poncirus trifoliata
Drought tolerance
Reactive oxygen species
Antioxidant enzymes

ABSTRACT

Drought is a major environmental factor that limits plant growth and productivity. Polyamines have been shown to act as stress molecules that accumulate in plant adaptation to abiotic stresses. In this study, an arginine decarboxylase gene isolated from *Poncirus trifoliata*, *PtADC*, was introduced into tobacco and tomato to investigate its function in drought tolerance. We demonstrate that the transgenic plants showed an improvement in dehydration and drought tolerance. Under dehydration stress conditions, the accumulation of reactive oxygen species (ROS) was remarkably decreased in the transgenic lines as compared with the wild type. Moreover, the transcript levels of three stress-responsive genes were increased in the transgenic tobacco lines. Taken together, our results suggest that *PtADC* plays a key role in drought tolerance, which is, at least partially, attributed to its role in ROS detoxification.

1. Introduction

Drought is one of the most devastating abiotic stresses that impacts growth and development and productivity of agricultural crops worldwide. It results in an array of physiological and biochemical alterations in the stressed cells, among which is the excessive accumulation of reactive oxygen species (ROS). ROS is detrimental to plants because it can cause lipid peroxidation, denaturation of proteins and nucleic acids [1]. Therefore, the rationale is that drought damage can be attenuated by means of inhibiting ROS accumulation.

The steady-state levels of ROS depend upon the physiological balance of production and scavenging. Scavenging of ROS is accomplished by a large spectrum of enzymatic and non-enzymatic antioxidants, including the polyamines (PAs). The PAs, mainly diamine putrescine (Put), triamine spermidine (Spd), and tetraamine spermine (Spm), are low-molecular-weight organic cations that are ubiquitously distributed in living organisms [2]. Accumulating evidences indicate that PAs are involved in a wide range of physiological processes, such as growth, development and abiotic stress response [3–5].

Two pathways are responsible for synthesis of Put in higher plants, a direct one catalyzed by ornithine decarboxylase and an indirect one by arginine decarboxylase (ADC, EC 4.1.1.19). The PA biosynthesis is primarily regulated at the transcriptional level, although conjugation and oxidase-mediated catabolism also participate in the regulation of endogenous PA levels [6]. In addition, PA biosynthesis consists of a series of catalytic steps, but the ADC-mediated one plays a predominant role in the accumulation of PAs under stresses [7–11]. With this in mind, it may be a feasible way to enhance the PA level by enhancing the mRNA level of *ADC* gene that regulates the activity of the PA biosynthesis.

Although *ADC* genes have been cloned from various plant species, the knowledge on its role in drought tolerance is still far from being fully explored. Moreover, the ROS accumulation in the transgenic plants overexpressing the *ADC* genes has been scarcely investigated. To address these issues, in the current study, we generated transgenic tobacco and tomato plants overexpressing an *ADC* gene (*PtADC*) isolated from *Poncirus trifoliata* [11]. Our data show that *PtADC* significantly increased tolerance to dehydration and drought in tobacco and tomato. In addition, histochemical staining revealed that ROS accumulation in the transgenic plants was remarkably impaired when compared with the wild type (WT).

2. Materials and methods

2.1. Plant materials and vector

Tomato (*Solanum lycopersicum* L. 'Zhongshu No. 4') and tobacco (*Nicotiana nudicaulis*) seeds were surface-sterilized with 75% (v/v) ethanol for 30 s, washed with sterilized water for 10 s and then incubated in a 2% NaClO for 15 min. After rinse with sterilized water, the seeds were sown on germination medium (Supplementary Table 1)

Abbreviations: ADC, arginine decarboxylase; PA, polyamine; DAB, 3,3′-diaminobenzidine; NBT, nitroblue tetrazolium; ROS, reactive oxygen species.

* Corresponding author.
E-mail address: liujihong@mail.hzau.edu.cn (J.-H. Liu).

[1] These two authors contribute equally to this work.

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.08.015

and cultured in a growth chamber (25 °C, 16 h light/8 h dark cycle, 72 μmol/m^2/s). One- or nine-week-old leaves collected from the tomato or tobacco *in vitro* seedlings were subjected to *Agrobacterium tumefaciens*-mediated transformation using the *Cauliflower Mosaic Virus* (*CaMV*) *35S-PtADC* construct [11].

2.2. Tobacco and tomato transformation

Leaf disc transformation was carried out essentially as previously described [12,13]. Transgenic plants were cultured on selection medium (Supplementary Table 1) containing kanamycin (km) as selective marker. km-resistant shoots were transferred to rooting medium. Putative transgenic T0 plants were kept in a growth chamber at 25 °C with a 16 h light/8 h dark photoperiod until they flowered and set seeds. Homozygous lines were harvested according to an earlier method [14].

2.3. Molecular confirmation of the transgenic plants

Genomic DNA was extracted from *in vitro* T0 plants and the WT using cetyltrimethyl ammonium bromide (CTAB). The 20-μL PCR reaction solution contained 2 μL of 10 × PCR buffer, 2 μL of 2.5 mM dNTPs, 0.5 μL of each primers (10 mM), and 2.5 units of *Taq* polymerase (TransGen Biotech, China). The PCR procedure included an initial 5-min denaturation at 94 °C, followed by 35 cycles of 94 °C for 35 s, 56 °C for 35 s, 72 °C for 60 s (for *NPTII*) or 90 s (for *CaMV*35S-*PtADC*), with a final 10-min extension at 72 °C. Primers used for the PCR reaction were the same as those described previously [11]. The PCR products were size-separated on 1.0% (w/v) agarose gel, which was stained with 0.5 μg/mL ethidium bromide (EB) for 10 min. The EB-stained gel was then visualized under UV transillumination and photographed.

2.4. RNA isolation and semi-quantitative RT-PCR

mRNA levels of *PtADC* in tobacco and tomato transgenic lines and three stress-responsive genes in tobacco were determined by semi-quantitative RT-PCR analysis. Total RNA was prepared using TRIZOL reagent (TaKaRa, Dalian, China) according to the manufacturer's protocol. The total RNA was treated with amplification-grade DNaseI (Takara, Dalian, China) to removed genomic DNA. cDNA was synthesized using ReverTra Ace-α-® First Strand cDNA Synthesis Kit (TOYOBO, FSK-100) following the manufacturer's instructions. RT-PCR reaction preparation and amplification were performed following the protocol described in [11].

2.5. Quantification of endogenous free polyamine contents in the transgenic plants

Free polyamines were extracted and measured by high performance liquid chromatography (HPLC) as described before [15]. Quantification of free polyamines was performed in three triplicates for each line.

2.6. Stress tolerance assay of the transgenic plants

Surface-sterilized seeds of the transgenic lines and WT were sown as mentioned above. Three-month-old potted plants of tomato and two-month-old *in vitro* plants of tobacco were used for dehydration treatment. Uniform and healthy leaves were excised from the plants, placed on dry filter papers and allowed to dehydrate for as long as 80 min (tobacco) or 360 min (tomato). Fresh weight (FW) of the leaves was measured at 10 or 30-min intervals to determine the rate of water loss in tobacco and tomato, respectively. Ion leakage (IL) and accumulation of O_2^- and H_2O_2 in the leaves were examined at the beginning and completion of dehydration as described below. Total chlorophyll content in the tomato plants was also measured at the end of dehydration.

For drought experiment, eight-week-old transgenic tobacco lines and the WT grown in a greenhouse were depleted of irrigation for 4 d. As for tomato, ten-week-old transgenic plants and WT were subjected to water withholding for 10 d on an experiment bench in the lab. All of the plants were regularly watered before drought treatment. Morphological changes of the droughted plants were inspected during the drought treatment. Photographs were taken when the differences were noticeable.

2.7. Measurement of ion leakage (IL), chlorophyll content and ROS staining

IL measurement was performed as previously described [4]. Total chlorophyll content was measured based on a spectrophotometric method [16]. *In situ* accumulation of O_2^- and H_2O_2 in the dehydrated leaves was investigated by histochemical staining with nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB), respectively [17].

2.8. Statistical analysis

Dehydration treatment was repeated three times with consistent results, and the results of a representative experiment were presented, shown as mean ± SE. Drought stress was repeated twice, and the phenotype of one experiment was shown. The data were analyzed using analysis of variance (ANOVA) by SAS software (version 8.0, SAS Institute, NC, USA). Statistical difference was compared based on Fisher's LSD test, at significance level of $P < 0.05$.

3. Results and discussion

3.1. Transformation and regeneration of transgenic tobacco and tomato plants

In our earlier work, *PtADC* was shown to be induced by dehydration stress [11]. To clarify if this gene functions in dehydration and drought tolerance, efforts were made to generate transgenic tobacco and tomato plants overexpressing *PtADC*. Tobacco and tomato transformation gave rise to a total of 14 and 12 km-resistant T0 plants, which were confirmed as putative transgenic origin by genomic PCR analysis (data not shown). Semi-quantitative RT-PCR analysis showed that mRNA level of *PtADC* was detected in two T2 lines selected from the populations (Fig. 1A and B). The overexpression lines did not display any morphological difference from the WT under normal growth conditions in this experiment.

3.2. Free polyamine levels in the transgenic lines

HPLC assay showed that free Put level in the tobacco transgenic lines was increased to 5.26 (#48) and 2.39 (#71) times that of the WT (Fig. 1C). Spd and Spm in #48 were higher than, but not statistically different, those of the WT, whereas their level in #71 was equivalent to the WT. The transgenic plants of tomato contained 26.3–39.5% higher Put level than the WT (Fig. 1D). Spd level was elevated by 26.2% in line #51 and 15.7% in line #55 as compared with the WT, but only the difference between #51 and WT reached significant level ($P < 0.05$). Nevertheless, there was no difference in free Spm level between the transgenic and WT plants. These data showed that overexpression of *PtADC* caused elevation of the endogenous Put level, implying that the transgene functions normally to synthesize Put in both tobacco and tomato. However, it is worth mentioning that the magnitude of Put increase varies depending on plant species since greater elevation of Put level

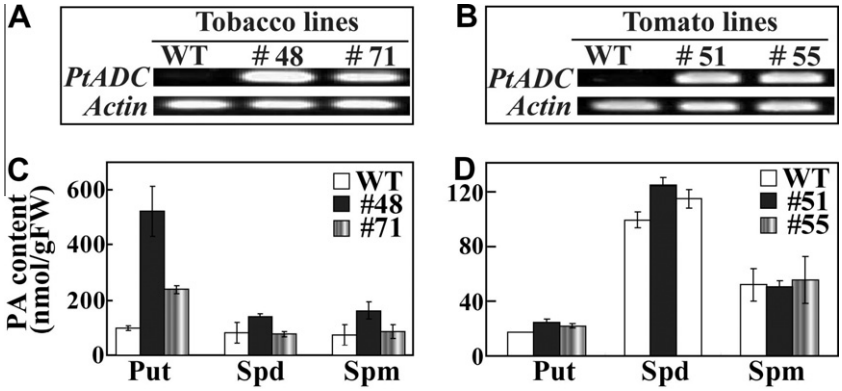

Fig. 1. Analysis of transgene expression and polyamine content. (A and B) *PtADC* expression level in transgenic lines of tobacco (A) and tomato (B), as analyzed by RT-PCR analysis using *Actin* as an internal content. (C and D) HPLC assay of free polyamine contents in tobacco (C) and tomato (D). Data shown are mean ± SE ($n = 3$).

was noticed in tobacco than in tomato. On the other hand, the tomato transgenic lines exhibited very limited increase of the Put level despite the overexpression of the transgene. Consistent with our finding, slight increase of the end products of a PA biosynthetic gene has been previously reported [9,11,18]. Interestingly, it is noticeable that although the tobacco transgenic lines #48 and #71 displayed prominent increase of the Put level, a concurrent rise of Spd and Spm was only found in the former line. Our results are in line with the previous report [19], but the underlying reason remains to be elucidated. One speculation is that endogenous Put pool in line #71 does not reach a critical threshold that is required to trigger the conversion of Put to downstream Spd and Spm [9].

3.3. Overexpression of PtADC confers tolerance to dehydration in tobacco and tomato

When the two-month-old leaves were subjected to dehydration in the ambient environment, steady water loss was detected in both the WT and transgenic lines. However, it is evident that the transgenic lines exhibited much less water loss relative to the WT at the same time point. After 80 min of dehydration the water loss rate of the two transgenic lines was 23.6% (#48) and 33.0% (#71), compared to 48.1% in the WT (Fig. 2A). Morphological differences were apparent as the transgenic leaves largely remained their turgor, but the WT leaves withered seriously (Fig. 2B). Likewise, the tomato transgenic lines lost water slower than the WT. For instance, the WT lost 27.0% of its water at the end of dehydration, but the water loss was 21.0% in #55 and 17.8% in #51 (Fig. 3A). Moreover, turgor of the transgenic leaves was maintained to the better degree relative to the WT (Fig. 3B).

Ion leakage (IL) and chlorophyll content are typical physiological indices for evaluating abiotic stress tolerance in crop plants [20,21]. IL has been extensively used as an indicator of plasma membrane damage under abiotic stresses [4,11]. As shown in Fig. 2C and Fig. 3C, there was no difference in IL between the WT and transgenic lines prior to the dehydration. Dehydration for 80 min or 360 min resulted in dramatic increase of IL in tobacco and tomato, respectively. Nevertheless, IL of the transgenic lines is remarkably lower than that of the WT. In addition, total chlorophyll content of the two tomato transgenic lines was 8.2 μg/gFW and 8.6 μg/gFW, higher than that of the WT (7.0 μg/gFW, Fig. 3D).

3.4. Overexpression of PtADC confers tolerance to drought in transgenic plants

A short-term (4 d) water depletion of the tobacco WT plants in the greenhouse led to conspicuous leaf withering, whereas the withering symptom was less serious in the two transgenic lines (Fig. 2D). When the tomato WT plants were exposed to drought for 10 d on the experimental bench, they displayed pronounced phenotypes associated with drought stress, such as leaf withering and shoot drooping. By contrast, the transgenic lines were less seriously affected by the drought under the same situations (Fig. 3D). After water was resumed, the growth of the transgenic lines was recovered quicker compared to the WT (Fig. 3E).

The above data clearly suggest that overexpression of *PtADC* rendered the tomato and tobacco transgenic plants more tolerant to dehydration and drought stress, which provides cogent evidence for the function of *PtADC* in the stress tolerance. Before this work, several studies have shown that transformation of *ADC* genes led to enhanced tolerance to drought [8,9,11,19], high osmoticum and low temperature [11]. Taken together, these results demonstrate that *ADC* is a potentially valuable gene that can be utilized in transgenic breeding to improve abiotic stress tolerance.

3.5. Less accumulation of ROS in the transgenic plants under dehydration

All the above results indicate that the transgenic lines suffered from less serious membrane damage compared to the WT. Since ROS level is associated with the membrane integrity, ROS accumulation in the leaves under dehydration was assessed by histochemical staining with NBT (for O_2^-) and DAB (for H_2O_2), which allows for subcellular localization of the ROS [22]. Under normal growth conditions, very light staining of the leaves was observed, implying that ROS accumulated at low levels. Meanwhile, no obvious difference was detected between the WT and transgenic lines. Exposure to dehydration led to extensive staining of the leaves in the WT and transgenic plants. Nevertheless, it is apparent that the WT accumulated greater quantities of ROS than the transgenic lines in tobacco (Fig. 4A and B) and tomato (Fig. 4C and D).

It is known that homeostasis of ROS is destroyed under adverse conditions, giving rise to greater accumulation of ROS and triggers oxidative stress [23]. Herein, the transgenic lines contained lower level of ROS under dehydration relative to the WT. This means that oxidative stress was weaker in the transgenic plants than in the WT, which is correlated with the enhanced stress tolerance of the former. The decreased level of ROS in the transgenic lines may be due to the higher free PA content. This assumption is not impossible since PAs have been shown to regulate ROS homeostasis by way of two possible modes of action. First, PAs form a ternary complex through the interaction with metal ions and the phospholipid polar head, leading to the inhibition of metal ion auto-oxidation and subsequent supply of electron for ROS

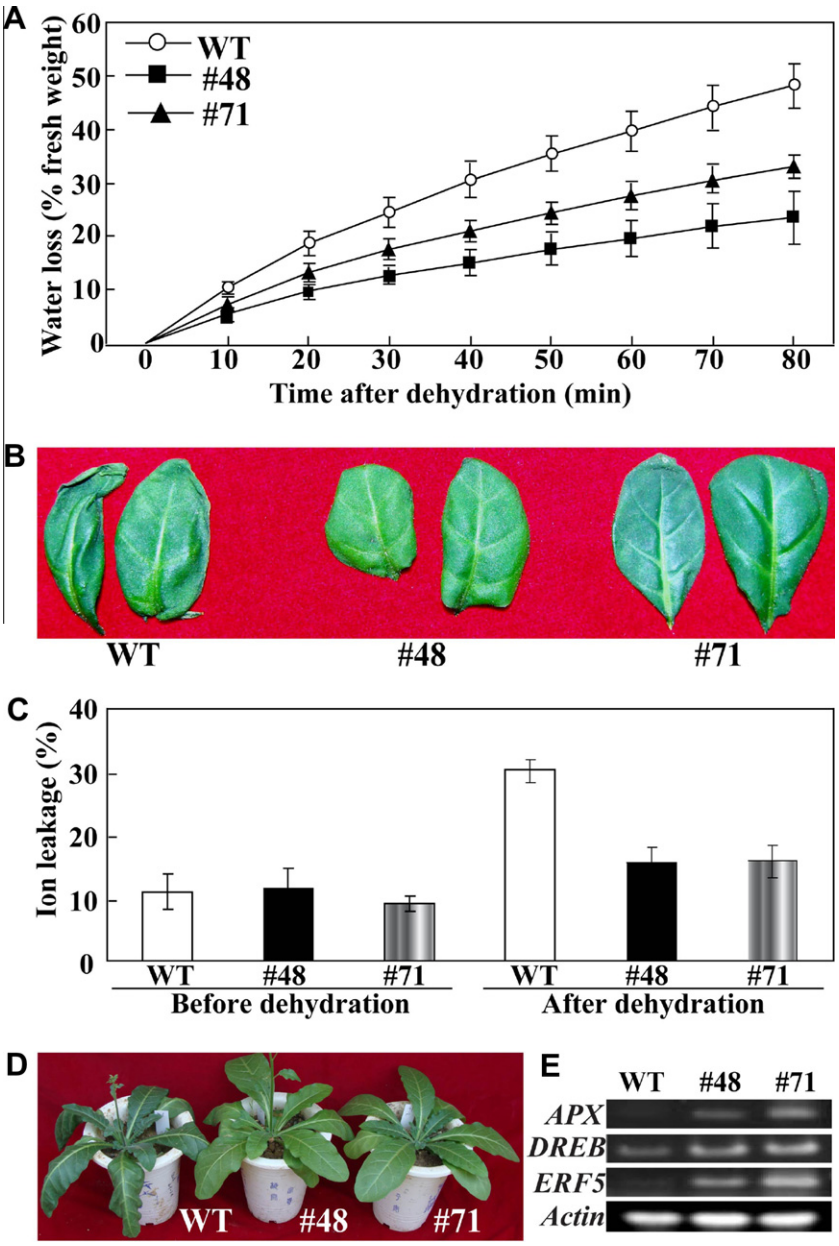

Fig. 2. Response of tobacco lines (#48 and #71) overexpressing *PtADC* to dehydration and drought stress. (A) Leaf water loss in the WT and transgenic lines under dehydration at ambient environment. (B) A representative photograph showing the WT and transgenic lines after 80 min of dehydration. (C) Ion leakage in the WT, #48 and #71 before and after 80-min dehydration. (D) A representative photograph showing the WT and transgenic lines after 4 d of drought. (E) RT-PCR analysis of stress-responsive genes, *APX*, *DREB* and *ERF5*, in the WT, #48 and #71 without stress.

generation [24]. This represents a direct role of PA in reducing ROS generation under stress. Second, as an indirect role, PAs activate the antioxidant enzymes, as has been demonstrated by a number of studies using exogenously supplied PAs or genetic approach [17,25–28]. In this regard, the stress-derived ROS may be subjected to more robust detoxification, leading to a lower level of ROS accumulation.

3.6. PtADC activates the expression of stress-responsive genes in tobacco

It has been suggested that gene expression levels are likely to be correlated with the magnitude of stress tolerance in plants [29]. To elucidate the molecular mechanism underlying the role of *PtADC* in abiotic stress response, expression patterns of three stress-related genes (*APX*, *DREB*, *ERF5*) were compared between the WT and the transgenic lines of tobacco growing under normal conditions. As can be seen in Fig. 2E, transcript levels of these genes were evidently higher in the transgenic lines than in the WT. It suggests that *PtADC* may function in abiotic stress tolerances through regulating or interacting with the stress-related genes, although it remains unclear how such regulation or interaction can be established. However, the induction of these stress-related genes may, at least in part, account for the better stress tolerance in the transgenic plants at transcriptional levels. *APX* gene encodes the ascorbate peroxidase that is a key antioxidant enzyme for the detoxification of H_2O_2 [30]. Stronger induction of *APX* gene indicates that the transgenic lines might possess a more powerful capacity of scavenging H_2O_2 in comparison with the WT, even in the absence of stresses. This is in agreement with the lower accumulation of H_2O_2 in the transgenic lines. ERF (ethylene response factors) subfamily transcription factors belong to the

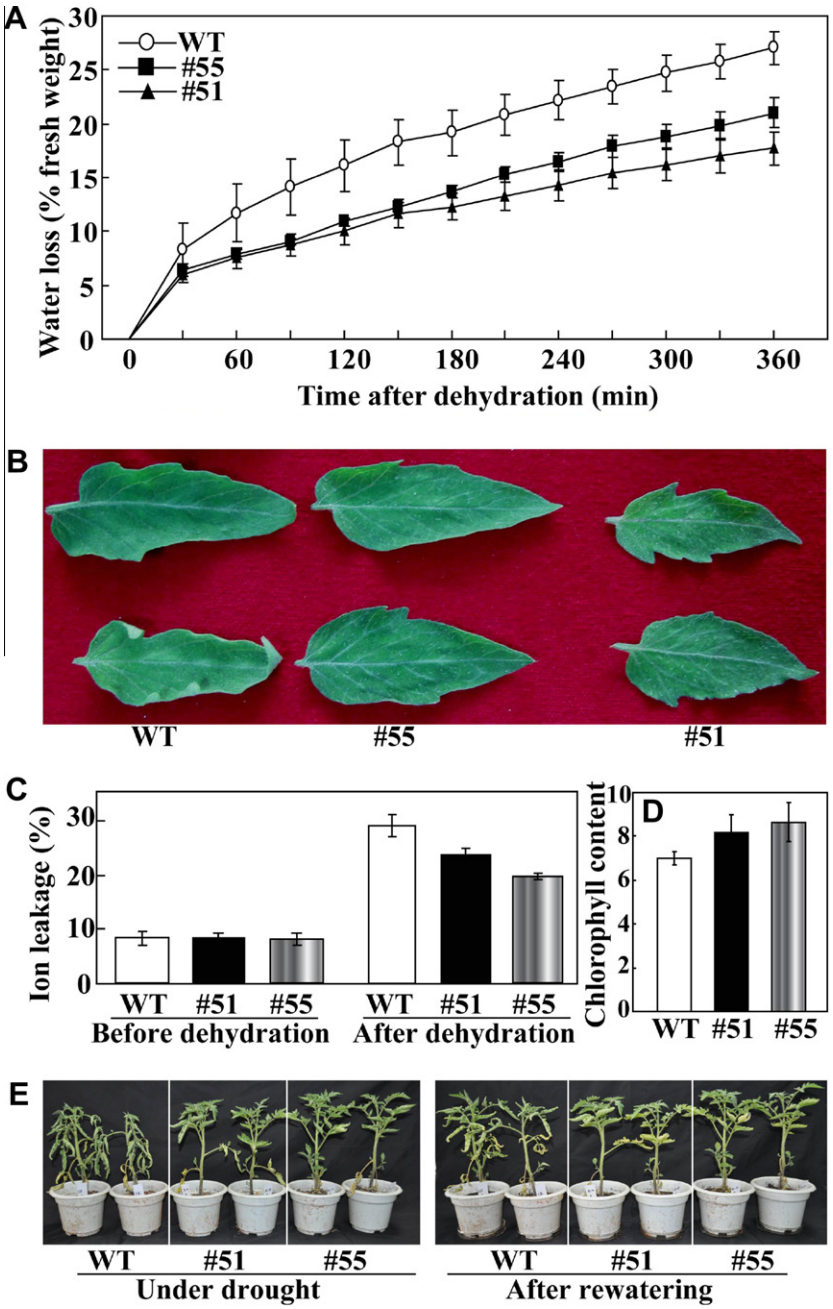

Fig. 3. Response of tomato lines (#51 and #55) overexpressing *PtADC* to dehydration and drought stress. (A) Leaf water loss in the WT and transgenic lines under dehydration at ambient environment. (B) A representative photograph showing the dehydrated leaves of WT and transgenic lines after 360 min of dehydration. (C) Ion leakage in the WT, #51 and #55 before and after dehydration. (D) Total chlorophyll in the dehydrated leaves of the WT, #51 and #55 collected at the end of dehydration. (E) A representative photograph showing the WT and transgenic lines after 10 d of water withholding (left panel), and water resumption for 6 h (right panel).

APETALA2(AP2)/ERF superfamily containing a highly conserved AP2/ERF binding domain. A growing body of work has demonstrated that ERF proteins are implicated in plant response to abiotic stresses [31–33]. Overexpression of an ERF transcription factor, *JERF3*, decreased ROS accumulation in tobacco transgenic plants under various stresses [33]. Of note, the expression of *APX* gene was enhanced in the transgenic plants expressing *JERF3*, in accordance with the larger induction of *APX* herein. These data suggest that activation of the ERF transcription factor by *PtADC* may be a part of the integrated protective machinery against the oxidative stress. *DREBs* are important transcription factors that can regulate the expression of a large spectrum of target genes, collectively known as a regulon, by binding to the *cis*-acting element in their promoters [34]. Many genes in the *DREB* regulon code for functional proteins that play direct roles in rendering stress tolerance [31,35]. Higher expression level of the *DREB* gene in the transgenic lines compels us to speculate that the regulons of the *DREB* may be efficiently mobilized. This, in turn, leads to production of more abundant metabolites that might act together to protect the cells against the stress.

Taken together, we show here that genetic transformation of a stress-inducible *PtADC* elevated dehydration and drought tolerance in tomato and tobacco. These results provide compelling evidence that *PtADC* plays an essential role in stress tolerance and holds great potential for engineering drought tolerance in cultivated plants. The enhanced stress tolerance might be correlated with

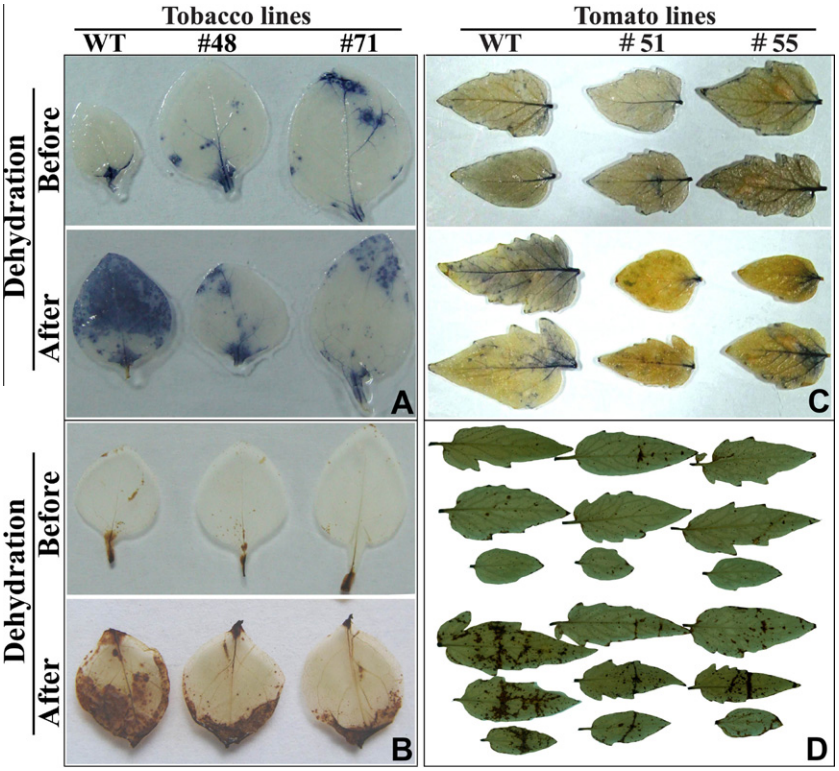

Fig. 4. Accumulation of O_2^- and H_2O_2 in the WT and transgenic lines before and after dehydration. (A–D) Representative photos showing staining with NBT (O_2^-, A, C) and DAB (H_2O_2, B, D) in tobacco (A, B) and tomato (C, D) leaves before (upper panel) and after dehydration (lower panel).

the higher levels of cellular PAs, which, on the one hand, repress accumulation of ROS and subsequently mitigate the oxidative stresses. On the other hand, expression profiles of stress-associated genes are altered towards a situation favorable for defense against the stresses. In the future, it will be significant to decipher the molecular events underlying upregulation of the genes, represented by several identified herein, which will gain deeper understanding on the mechanism of action of *PtADC* in stress tolerance.

Acknowledgments

This research was financially supported by the National Natural Science Foundation of China (30871685, 30921002, 31071778), the Research Fund for the Doctoral Program of Higher Education (20090146110010), Fok Ying Tong Education Foundation (114034), the National High Technology Research and Development Program (863 Program) of China (2011AA100205), and Wuhan Municipal Project for Academic Leaders (201150530148). The authors are grateful to Prof. Yue-Peng Han for critical reading of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.08.015.

References

[1] C.J. Hansen, L. Esposito, A.I.F. Stewart, J. Colwell, A. Hendrix, W. Pryor, D. Shemansky, R. West, Enceladus' water vapor plume, Science 311 (2006) 1422–1425.

[2] J. Martin-Tanguy, Metabolism and function of polyamines in plants: recent development (new approaches), Plant Growth Reg. 34 (2001) 135–148.

[3] P.T. Evans, R.L. Malmberg, Do polyamines have roles in plant development, Annu. Rev. Plant Physiol. Plant Mol. Biol. 40 (1989) 235–269.

[4] J.H. Liu, K. Nada, C. Honda, H. Kitashiba, X.P. Wen, X.M. Pang, T. Moriguchi, Polyamine biosynthesis of apple callus under salt stress: importance of the arginine decarboxylase pathway in stress response, J. Exp. Bot. 57 (2006) 2589–2599.

[5] R. Alcázar, T. Altabella, F. Marco, C. Bortolotti, M. Reymond, C. Koncz, P. Carrasco, A.F. Tiburcio, Polyamines: molecules with regulatory functions in plant abiotic stress tolerance, Planta 231 (2010) 1237–1249.

[6] H.J. Rhee, E.J. Kim, J.K. Lee, Physiological polyamines: simple primordial stress molecules, J. Cell. Mol. Med. 11 (2007) 685–703.

[7] J.H. Liu, H. Kitashiba, J. Wang, Y. Ban, T. Moriguchi, Polyamines and their ability to provide environmental stress tolerance to plants, Plant Biotechnol. 24 (2007) 117–126.

[8] M. Roy, R. Wu, Arginine decarboxylase transgene expression and analysis of environmental stress tolerance in transgenic rice, Plant Sci. 160 (2001) 869–875.

[9] T. Capell, L. Bassie, P. Christou, Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress, Proc. Natl. Acad. Sci. USA 101 (2004) 9909–9914.

[10] K. Urano, Y. Yoshiba, T. Nanjo, T. Ito, K. Yamaguchi-Shinozaki, K. Shinozaki, *Arabidopsis* stress-inducible gene for arginine decarboxylase *AtADC2* is required for accumulation of putrescine in salt tolerance, Biochem. Biophys. Res. Commun. 313 (2004) 369–375.

[11] J. Wang, P.P. Sun, C.L. Chen, Y. Wang, X.Z. Fu, J.H. Liu, An arginine decarboxylase gene *PtADC* from *Poncirus trifoliata* confers abiotic stress tolerance and promotes primary root growth in *Arabidopsis*, J. Exp. Bot. 62 (2011) 2899–2914.

[12] R.B. Horsch, J.E. Fry, N.L. Hoffman, D. Eicholtz, S.G. Rogers, R.T. Fraley, A simple and general method for transferring genes into plants, Science 227 (1985) 1229–1231.

[13] H.J. Sun, S. Uchii, S. Watanabe, H. Ezura, A highly efficient transformation protocol for Micro-Tom, A model cultivar for tomato functional genomics, Plant Cell Physiol. 47 (2006) 426–431.

[14] J.H. Liu, Y. Ban, X.P. Wen, I. Nakajima, T. Moriguchi, Molecular cloning and expression analysis of an arginine decarboxylase gene from peach (*Prunus persica*), Gene 429 (2009) 10–17.

[15] X.B. Wu, J. Wang, J.H. Liu, X.X. Deng, Involvement of polyamine biosynthesis in somatic embryogenesis of Valencia sweet orange (*Citrus sinensis*) induced by glycerol, J. Plant Physiol. 166 (2009) 52–62.

[16] J.H. Liu, H. Inoue, T. Moriguchi, Salt stress-mediated changes in free polyamine titers and expression of genes responsible for polyamine biosynthesis of apple *in vitro* shoots, Env. Exp. Bot. 62 (2008) 28–35.

[17] J. Shi, X.Z. Fu, T. Peng, X.S. Huang, Q.J. Fan, J.H. Liu, Spermine pretreatment confers dehydration tolerance of citrus in vitro plants *via* modulation of

antioxidative capacity and stomatal response, Tree Physiol. 30 (2010) 914–922.

[18] A. Peremarti, L. Bassie, P. Christou, T. Capell, Spermine facilitates recovery from drought but does not confer drought tolerance in transgenic rice plants expressing *Datura stramonium S*-adenosylmethionine decarboxylase, Plant Mol. Biol. 70 (2009) 253–264.

[19] R. Alcázar, J. Planas, T. Saxena, X. Zarza, C. Bortolotti, J. Cuevas, M. Bitrián, A.F. Tiburcio, T. Altabella, Putrescine accumulation confers drought tolerance in transgenic *Arabidopsis* plants over-expressing the homologous *Arginine decarboxylase 2* gene, Plant Physiol. Biochem. 48 (2010) 547–552.

[20] X. Mao, H. Zhang, S. Tian, X. Chang, R. Jing, TaSnRK2.4, an SNF1-type serine/threonine protein kinase of wheat (*Triticum aestivum* L.), confers enhanced multistress tolerance in *Arabidopsis*, J. Exp. Bot. 61 (2010) 683–696.

[21] X.S. Huang, T. Luo, X.Z. Fu, Q.J. Fan, J.H. Liu, Cloning and molecular characterization of a mitogen-activated protein kinase gene from *Poncirus trifoliata* whose ectopic expression confers dehydration/drought tolerance in transgenic tobacco, J. Exp. Bot. 62 (2011) doi:10.1093/jxb/err229.

[22] V. Shulaev, D.J. Oliver, Metabolic and proteomic markers for oxidative stress. New tools for reactive oxygen species research, Plant Physiol. 141 (2006) 367–372.

[23] O. Blokhina, E. Virolainen, K.V. Fagerstedt, Antioxidants, oxidative damage and oxygen deprivation stress: a review, Ann. Bot. 91 (2003) 179–194.

[24] B. Tadolini, Polyamine inhibition of lipoperoxidation. The influence of polyamines on iron oxidation in the presence of compounds mimicking phospholipid polar heads, Biochem. J. 249 (1988) 33–36.

[25] M.D. Groppa, M.L. Tomaro, M.P. Benavides, Polyamines as protectors against cadmium or copper-induced oxidative damage in sunflower leaf discs, Plant Sci. 161 (2001) 481–488.

[26] L.X. He, Y. Ban, H. Inoue, N. Matsuda, J. Liu, T. Moriguchi, Enhancement of spermidine content and antioxidant capacity in transgenic pear shoots overexpressing apple spermidine synthase in response to salinity and hyperosmosis, Phytochemistry 69 (2008) 2133–2141.

[27] X.P. Wen, Y. Ban, H. Inoue, N. Matsuda, T. Moriguchi, Spermidine levels are implicated in heavy metal tolerance in a *spermidine synthase* overexpressing transgenic European pear by exerting antioxidant activities, Transgenic Res. 19 (2009) 91–103.

[28] J.C. Yiu, C.W. Liu, D.Y.T. Fang, Y.S. Lai, Waterlogging tolerance of Welsh onion (*Allium fistulosum* L.) enhanced by exogenous spermidine and spermine, Plant Physiol. Biochem. 47 (2009) 710–716.

[29] M.A. Carvallo, M.T. Pino, Z. Jeknić, C. Zou, C.J. Doherty, S.H. Shiu, T.H.H. Chen, M.F. Thomashow, A comparison of the low temperature transcriptomes and CBF regulons of three plant species that differ in freezing tolerance. *Solanum commersonii*, *Solanum tuberosum*, and *Arabidopsis thaliana*, J. Exp. Bot. 11 (2011) 3807–3819.

[30] C.A. Jaleel, K. Riadh, R. Gopi, P. Manivannan, J. Inès, H.J. Al-Juburi, C.X. Zhao, H.B. Shao, R. Panneerselvam, Antioxidant defense response: physiological plasticity in higher plants under abiotic constraints, Acta Physiol. Plant. 31 (2009) 427–436.

[31] P.K. Agarwal, P. Agarwal, M.K. Reddy, S.K. Sopory, Role of DREB transcription factors in abiotic and biotic stress tolerance in plants, Plant Cell Rep. 25 (2006) 1263–1274.

[32] Z.J. Zhang, F. Li, D.J. Li, H.W. Zhang, R.F. Huang, Expression of ethylene response factor *JERF1* in rice improves tolerance to drought, Planta 232 (2010) 765–774.

[33] L. Wu, Z. Zhang, H. Zhang, X.C. Wang, R. Huang, Transcriptional modulation of ethylene response factor protein JREF3 in the oxidative stress response enhances tolerance of tobacco seedlings to salt drought, and freezing, Plant Physiol. 148 (2008) 1953–1963.

[34] K. Shinozaki, K. Yamaguchi-Shinozaki, Gene networks involved in drought stress response and tolerance, J. Exp. Bot. 58 (2007) 221–227.

[35] C. Lata, M. Prasad, Role of DREBs in regulation of abiotic stress response in plants, J. Exp. Bot. 10 (2011) 1–18.