Pak. J. Bot., 52(3): 793-799, 2020. DOI: http://dx.doi.org/10.30848/PJB2020-3(32)

PRODUCTION OF DROUGHT TOLERANT TRANSGENIC SOYBEAN EXPRESSING *cod*A GENE UNDER REGULATION OF A WATER STRESS INDUCIBLE PROMOTER

DONG THI TA[1+], DUNG MANH NGO[3+], NHUNG HONG NGUYEN[1], NGOC BICH PHAM[1,2], PHAT TIEN DO[1] AND HA HOANG CHU[1,2*]

[1]*Institute of Biotechnology, Vietnam Academy of Science and Technology, 18-Hoang Quoc Viet, Cau Giay, Ha Noi, Vietnam*
[2]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology, 18-Hoang Quoc Viet, Cau Giay, Ha Noi, Vietnam*
[3]*Thai Nguyen University of Education, Thai Nguyen University, Luong Ngoc Quyen, Thai Nguyen, Vietnam*
[*]*Corresponding author's e-mail: chuhoangha@ibt.ac.vn*
[+]*These authors contributed equality to this work*

Abstract

Currently, drought stress has been known as a critical abiotic factors limiting crop production around the world. Several genes associated with water stresses, including *codA* gene encoded for choline oxidase, have been characterized and transferred to several important crops to enhance the tolerance to drought stress. Therefore, in this study, *codA* gene under the regulation of a drought inducible promoter-rd29A was introduced into soybean using the *Agrobacterium*-mediated method. The presence of *codA* gene in transgenic soybeans was confirmed by PCR and Southern hybridization. Under drought stress treatments, transgenic soybeans showed an enhanced seedling growth and plant biomass as compared to non-transformed soybeans. Moreover, the contents of glycine betaine and free proline, two major organic osmolytes as well as peroxidase activity were found increased in *codA* transgenic soybean. The malondialdehyde content in transformed soybeans decreased by less than 50% relative to control soybean plants under drought stress conditions. This is the first report on the utilization of the *codA* gene to improve drought tolerance in soybeans.

Key words: codA gene, Drought stress, Glycine betaine, Soybean transformation.

Introduction

Soybean [*Glycine max* (L.)] is one the most important food and feed crops cultivated worldwide, accounting for 71% of world protein meal consumption and approximately 61% of global vegetable oil production (Soystat, 2017). Similar to other important crops, soybean production is threatened by various abiotic stresses (Phang *et al*., 2008), in which drought has been considered the most critical stress for soybean production (Lenssen, 2012). Therefore, the improvement of drought tolerant capacity of soybean cultivars is being attracted for sustainable soybean production. Among various approaches used, application of genetic engineering has been known as an efficient and potential technology to develop abiotic stress-tolerant plant species.

Glycine betaine (GB) has been known as a particularly effective compound for protecting plants from osmotic stresses (Murata *et al*., 1992). This compound stabilizes bioactivity and structures of a range of enzymes and complex proteins in plant cells under stresses (Papageorgiou & Murata, 1995). Chen & Murata (2008) showed that under different abiotic stresses, the GB application helped to improve the plant growth and survival rate. The tolerant abilities to different abiotic stresses, such as salt, cold, and drought can be seen in plants genetically engineered with biosynthetic GB genes or transcription factors (Hayashi *et al*., 1997; Alia *et al*., 1998a; Alia *et al*., 1998b; Sakamoto &Murata, 2000; Sulpice *et al*., 2003; Amanda *et al*., 2014).

Of several important genes related to the GB biosynthesis pathway, the *codA* gene encoded for choline oxidase has been characterized and utilized for production of transgenic plant species to tolerate with environmental stresses. Transgenic Arabidopsis plants overexpressed the *codA* gene driven by the 35S promoter produced the high GB content and promoted the tolerant abilities to salt, hot and cold stresses (Hayashi *et al*., 1997; Alia *et al*., 1998b, Sakamoto *et al*., 2000). Such improvement in abiotic stress tolerance were found in transgenic tobacco and rice with overexpression of *codA* (Kathuria *et al*., 2009; Jingjiang *et al*., 2013). The increased GB accumulation coupled with the improvement in salt and water deficit tolerance was observed in *codA* transgenic alfalfa (Li *et al*., 2014).

Genetic engineering of soybeans for drought tolerant improvement has been reported in several publications. According to Li *et al*., (2017), transgenic soybean plants overexpressing *GmFDL19* gene were found to grow better in salt and drought conditions. Overexpression of the gene encoded for betaine aldehyde dehydrogenase (BADH) showed the increased tolerance of soybeans to water deficiency (Qin *et al*., 2017). In addition, drought tolerance was confirmed in transgenic soybeans that overexpress the *AtABF3* gene (Kim *et al*., 2018). However, there have been no report utilizing the *codA* gene for the drought tolerance in soybeans and neither of the previous studies employed inducible promoter to express the transgenes in this plant species. In this study, the plant expression vector harbouring *codA* gene regulated by the stress-inducible rd29A promoter was constructed and transformed into soybeans using *Agrobacterium*-mediated method. The integration of *codA* into transgenic soybean plants was confirmed by Southern blots. In addition, the effects of *codA* expression on seedling and plant growth under water deficient condition were analysed. Biochemical index, including GB, proline content, malondialdehyde (MDA) and peroxidase (POD) activity in transgenic soybeans was also analysed under drought stress conditions.

Materials and Methods

Trasngenic vector construction and soybean transformation: Soybean cultivar DT22 (Vietnam national cultivar) was provided by The Legumes Research and Development Center, Vietnam Academy of Agricultural Science. The *codA* nucleotide sequence of *Arthrobacter globiformis* from GenBank (SEQ ID NO: AY304485) was used for codon optimization for the expression in plants. Sequences for the transit peptide and a c-myc tag were added to the codon-optimized *codA* gene (Text supplemental data S1). The whole nucleotide sequence of targeted fragment was synthesized by Epoch Life Science Inc. (Missouri City, Texas, USA) and inserted into the binary vector pIBTII regulated by a water stress-inducible promoter r*d29A*. The designed transgenic vector was mobilized into *Agrobacterium tumefaciens* C58/pGV2206 (IPK, Gatersleben, Germany) for plant transformation. We used an improved *Agrobacterium*-mediated cotyledon node transformation procedure from previous report (Margie *et al*., 2004). Transgenic soybeans were screened and identified using the leaf painting method with a solution containing 250 mg/mL phosphothricin and 0.1% Tween-20 (Zhang *et al*., 1999). Transgenic plants were maintained in the greenhouse with a 16-h light/8-h dark photoperiods.

Genomic extraction and PCR analysis: The transgenic soybean leaves was used for genomic DNA extraction with the improved CTAB protocol (Dellaporta *et al*., 1983). Total genomic DNA and specific primers for the *codA* gene (codA_F: 5' GTGTTGCAGTTGGATCGTTG; codA_R: 5' CCCTACACCTGGAGAGTCAA) or *bar* gene (bar_F: 5' AAACCCACGTCATGCCAGTT; bar_R: 5' GACAAGCACGGTCAACTTCC) were used to identify putative transgenic plants. PCR conditions consisted of an initial denaturation at 94°C for 5 min, followed by 30 cycles of 20 sec at 94°C, 30 sec at 53°C, 1 min at 72°C, and ended with an extension step of 5 min at 72°C. Then, obtained PCR products were run on a 0.8% agarose gel stained with ethidium bromide and captured on UV transilluminator

Southern hybridization analysis: Purified genomic DNA of putative transgenic soybeans was digested by *Eco*RI restriction enzyme that has only one cutting site in the T-DNA region. The treated DNA was fragmented by 1.0% agarose gel electrophoresis and then transferred to a hybridization nylon membrane (Zeta-Probe® GT, Bio-Rad, Hercules, CA, USA). DNA was fixed on the nylon membrane uising UV cross-linking. A 500 bp PCR fragment, amplified using primers of the *codA* gene (codA-F: 5'- TCCAACAGGCGTAGCATTGT-3'; codA-R: 5'- AGGCCGTCTTCAGTAGGAGT-3'), was used as the probe for hybridization. The random Primer Labelling Kit (Prime-It® RmT, Stratagene, La Jolla, CA, USA) was utilized to generate the labelled ^{32}P probes followed manufacturer's protocol. Hybridization and membrane washing was performed in an oven at 65°C using the procedure from Zeta-Probe® GT manufacturer (Bio-Rad, Hercules, CA, USA).

Seed germination under stress conditions: Seeds of T3 generation were sterilized under chlorine gas (Di *et al*., 1996) and planted on Murashige and Skoog basal medium with B5 vitamins solidified with 2.5 g/l phytagel added different concentrations of PEG 8000 (0, 10%, 15%). The culture plates were placed at 26°C with a 16 h light and 8 h dark photoperiods. Root length and seedling height were recorded after 12 days on the treatment media.

Drought stress treatments: The T_3 progenies, derived from four independent transgenic plants, were individually grown in soil pots (115 x 85 x 105 mm), screened by a herbicide-resistant test and used to study drought stress following the procedure by Wright & Lenssen (2013). Briefly, T_3 transgenic soybeans at the V3 stage (with three leaves) were grown in the environmental chamber set at 30-33°C and humidity of 60%. For control conditions, T_3 and wild-type soybeans were grown and well-watered in the other chamber at 28°C and humidity of 80%. Both root and stem weights of the tested soybean plants were recorded on the 9th day of the stress treatment.

Biochemical analysis: Leaves from treated and non-treated plants were analysed for GB, MDA, free proline content, and antioxidant enzyme POD activity. Accumulated glycine betaine was determined using a previous protocol (Grieve & Grattan, 1983). The contents of MDA, free proline, and POD activity were determined as described by Chen & Zhang (2016). The crude protein measurement was conducted as described by Bradford (1976).

Data analysis: The data were analysed using SPSS software. The mean value and standard deviation (SD) were calculated from three experimental replicates. The responses of transgenic and wild-type lines were compared using t-test at p-value ≤ 0.05.

Results

Plant transformation and confirmation of transgene integration: We generated 23 independent transgenic events from three transformation experiments utilizing our improved *Agrobacterium*-mediated cotyledon node transformation procedure (Table S1). The presence of *codA* and *bar* genes was confirmed by herbicide leaf painting and PCR with specific primers (Fig. S1). To estimate the numbers of transgene loci and copy in the transgenic soybeans, we randomly selected four independent transgenic events for Southern blot analysis. The Southern blot result utilized the *codA* partial open-reading frame as a probe and revealed one or two hybridization bands (Fig. 1). This indicates the single insertion of the *codA* gene in events D3, D4, and D7 and two copies in the event D2. The identical insertion pattern of transgenic plants D3 and D4 indicated that these two plants may be generated from the same independent event. These four transgenic lines were grown and self-pollinated under greenhouse conditions to propagate seeds for further analysis. Under greenhouse conditions, transgenic and non-transgenic soybean plants exhibited no difference in plant morphology.

Table S1. Soybean transformation and confirmation of transgene integration.

Transformation No.	Infected cotyledons	Shoot induction	Formed shoots	Rooted plants on selection medium	PPT resistance	CodA and bar positive	Confirmed by Southern Blot
Expt 1	723	545	28	18	6	6	
Expt 2	815	621	39	18	7	7	
Expt 3	722	562	41	16	4	4	
Expt 4	765	497	32	17	6	6	
Total	3025	2225	140	69	23	23	4/4
No infection	100	100	0	0	0	0	0

Text supplemental data S1. The codA fragment used for synthesis by Epoch Life Science Inc.

ATGGCACAAATTAACAACATGGCACAAGGGATACAAACCCTTAATCCCAATTCCAATTTCCATAAACCCCAAGTTCCTA
AATCTTCAAGTTTTCTTGTTTTTGGATCTAAAAAACTGAAAAATTCAGCAAATTCTATGTTGGTTTTGAAAAAAGATTCA
ATTTTTATGCAAAAGTTTTGTTCCTTTAGGATTTCAGCATCAGTGGCTACAGCCTGCATGCACATCGATAATATTGAAAA
TCTTAGTGACAGAGAGTTCGACTACATTGTTGTTGGAGGTGGTTCAGCTGGAGCTGCAGTGGCTGCACGTCTTTCTGAA
GATCCTGCAGTTAGTGTTGCCTTGGTTGAAGCCGGACCTGATGACAGAGGGGTGCCAGAGGTGTTGCAGTTGGATCGTT
GGATGGAATTGCTTGAATCTGGATACGATTGGGATTATCCAATAGAACCACAAGAGAATGGCAATAGTTTTATGAGGC
ATGCACGTGCTAAAGTCATGGGCGGTTGTTCTTCACATAACAGTTGCATTGCATTCTGGGCCCCAAGGGAGGATTTGGA
TGAGTGGGAAGCAAAATATGGTGCTACTGGCTGGAATGCTGAGGCTGCATGGCCTCTTTACAAAAGGCTTGAAACTAA
CGAAGATGCAGGACCAGATGCTCCACATCACGGAGATTCAGGGCCTGTCCACTTGATGAATGTGCCACCTAAAGATCC
AACAGGCGTAGCATTGTTGGATGCATGCGAACAGGCCGGCATTCCTAGAGCCAAGTTTAATACCGGCACAACAGTCGT
TAATGGAGCCAACTTCTTTCAGATTAATAGAAGGGCTGACGGGACAAGGTCATCTTCAAGTGTCTCATATATCCATCCA
ATCGTCGAGCAAGAAAACTTTACTTTGTTGACTGGTCTTAGGGCACGTCAACTTGTTTTCGACGCTGATAGACGTTGCA
CTGGGGTTGATATAGTCGACTCTGCCTTTGGACATACACATAGGCTTACAGCTAGAAACGAAGTCGTACTTTCTACTGG
TGCAATTGATACACCAAAGTTGCTTATGTTGTCTGGAATCGGGCCTGCAGCACACCTTGCTGAACATGGAATCGAGGTG
TTGGTTGACTCTCCAGGTGTAGGGGAACACCTTCAAGATCACCCTGAAGGAGTTGTCCAGTTCGAGGCTAAACAACCAA
TGGTCGCCGAATCAACCCAATGGTGGGAGATCGGTATCTTTACTCCTACTGAAGACGGCCTTGATAGGCCAGACCTTAT
GATGCATTACGGTTCAGTCCCTTTCGATATGAATACTTTGAGACATGGGTATCCTACAACTGAGAATGGATTCTCATTG
ACCCCTAATGTTACACATGCCAGGTCTAGGGGTACTGTGAGACTTAGAAGTAGGGACTTTAGAGACAAGCCTATGGTTG
ATCCTAGATATTTCACCGACCCAGAAGGCCACGATATGAGAGTAATGGTGGCAGGTATTAGAAAGGCTAGAGAGATTG
CAGCTCAACCTGCTATGGCTGAATGGACCGGTAGAGAGCTTTCACCAGGAGTTGAGGCCCAAACCGATGAAGAATTGC
AGGATTATATTCGTAAGACTCATAACACCGTTTACCACCCTGTGGGTACCGTGAGAATGGGCGCTGTGGAAGACGAGA
TGAGTCCACTTGATCCTGAATTGAGAGTGAAAGGAGTGACAGGACTTAGGGTCGCAGATGCTTCAGTGATGCCTGAAC
ATGTAACAGTTAACCCTAATATCACCGTGATGATGATAGGTGAGCGTTGTGCTGATTTGATTAGATCAGCTAGAGCAGG
GGAGACTACCACCGCTGATGCTGAACTTTCTGCAGCTTTGGCACAGAAACTCATCTCAGAAGAGGATCTGAATTGA

The red shaded characters indicate nucleotide sequences of transit peptide of the Rubisco small subunit of tobacco
The yellow shaded characters indicate c-myc tag nucleotide sequences for the detection of recombinant *codA*

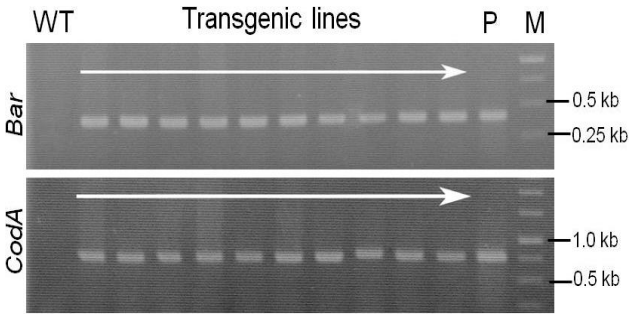

Fig. S1. Gel electrophoresis of the PCR product using specific primers for *codA* and *bar* genes. (WT) non transgenic wild-type soybeans; (P) positive control; (M) 1kb DNA ladder.

Soybean germination under the drought conditions: Seed germination and seedling establishment considered as critical stages for plant growth and development are specifically sensitive to water limitation conditions (Ahmad *et al*., 2008; Li *et al*., 2014). Therefore, transgenic soybeans at T_3 generation were evaluated in artificial drought conditions by supplementing 10% or 15% PEG 8000 into nutrient medium. On the 5th day on the control medium, no significant difference in the length of seedling was found in both *codA* expression, and the control soybean plants (Fig. 2a). All seeds showed good germination with seedling lengths of approximately 5 cm. By contrast, on treatment with 10% PEG 8000, non-transgenic soybean plants exhibited a significant reduction in seedling length (less than 4 cm), while transgenic soybean lines maintained normal seedling growth (Fig. 2b). Under higher stress conditions (15% PEG 8000), soybean growth was inhibited, resulting in dramatic decreases in the seedling lengths for both transgenic and wild-type plants. However, transgenic soybeans maintained a faster growth rate and showed significantly longer seedlings than non-transgenic plants did. The obtained results demonstrate that the ectopic *codA* gene expression improved the tolerance of soybean seedling growth against drought stress.

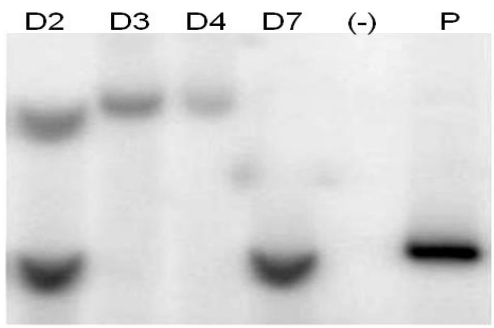

Fig. 1. Southern blot analysis of soybean transgenic lines. D2-D7: Transgenic lines; (-) non-transgenic wild-type line; P. positive control (binary vector pIBTII-Rd29a-TPCodA).

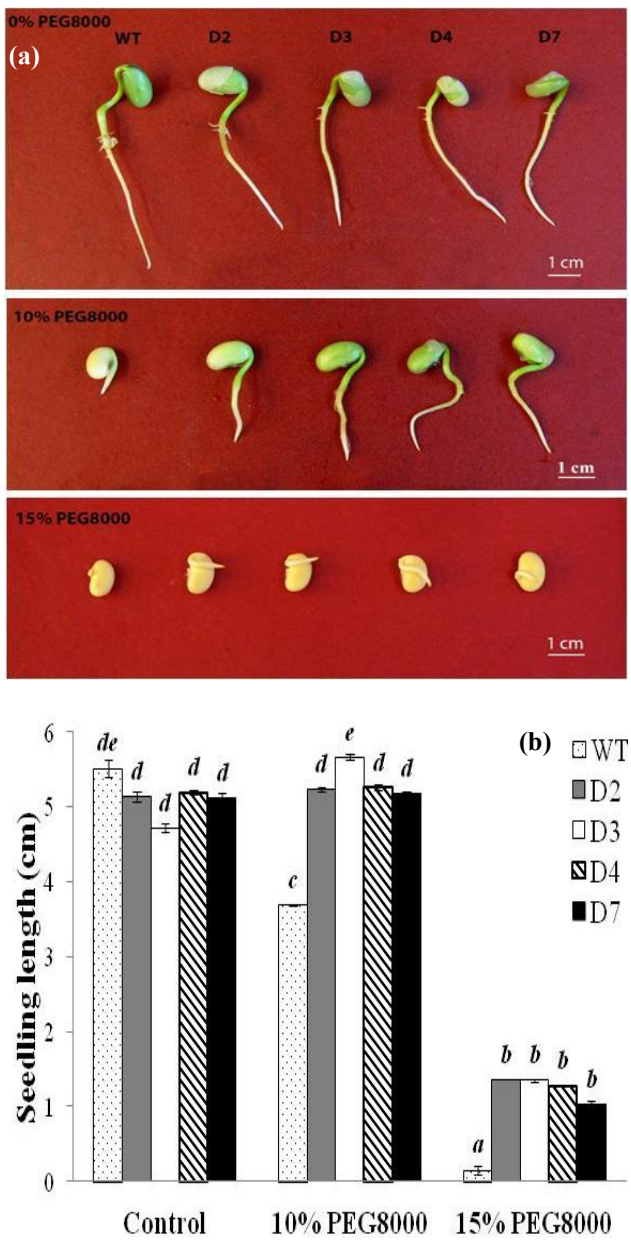

Fig. 2. Seed germination and seedling growth under drought stress treatments. (a) Germination of soybeans in MS medium (upper) and induced-drought medium with 10% PEG8000 (middle), 15% PEG8000 (lower) on the 12th days; (b) soybean seed lengths in different germination treatments. D2-D7: Transgenic soybean events; (WT) non-transgenic wild-type; Values denoted by different letters are significantly different at $p<0.05$.

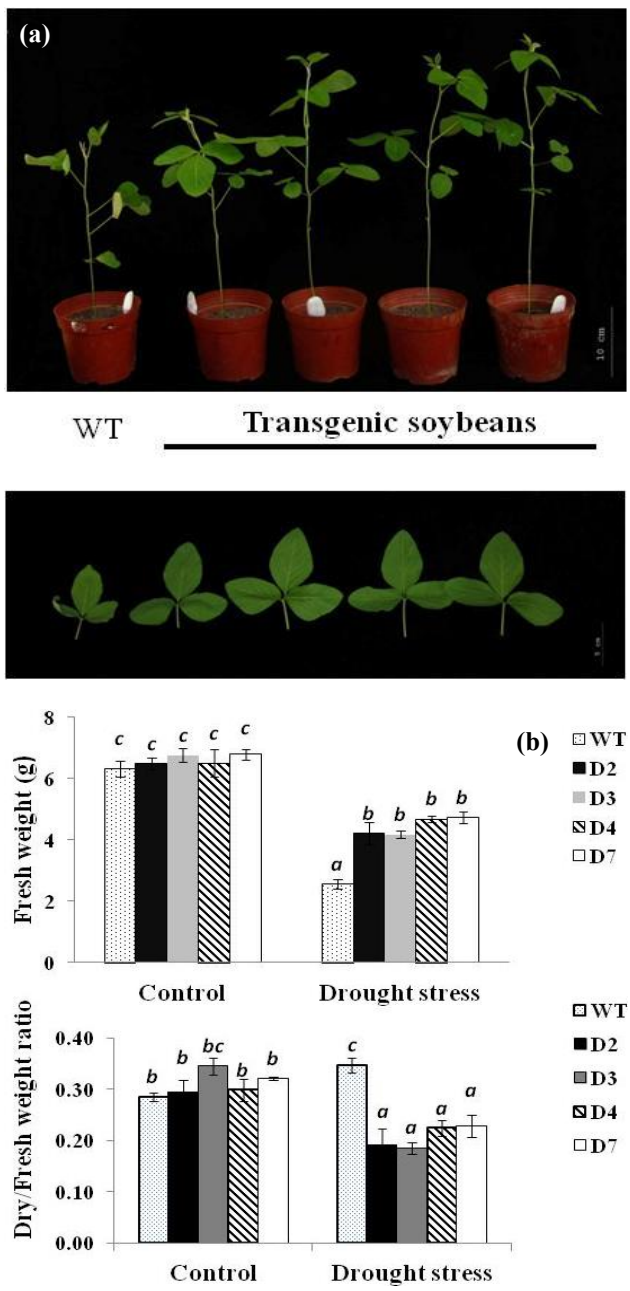

Fig. 3. Soybean growth and biomass production under drought stress conditions. (a) Soybean plants on the 9th day under water stress conditions (upper) and the leaflet phenotype (lower); (b) soybean fresh weight under well-watered and drought stress treatments; D2-D7: Transgenic soybean events; (WT) non-transgenic wild-type soybeans; Values denoted by different letters are significantly different at $p<0.05$.

Soybean growth under water deficiency: To evaluate soybean growth and development under drought stress, T_3 seeds of *codA* expression soybeans and non-transformed soybeans were planted individually in a pot and tested by leaf painting for herbicide resistance. At the V2 stage, soybean plants were exposed to different water available treatments in a growth chamber. Under a well-watered treatment condition, transgenic and wild-type soybean plants showed no difference in plant morphology (data not shown). In contrast, under water deficient conditions, the growth rate of wild-type soybean plants was decreased significantly compared to transgenic ones (Fig. 3a). On the 9th day under drought stress, non-transgenic soybeans showed severe symptoms, including yellow, dry and withered leaves, while although reduction in plant growth rate was occurred, no severe leaf damage was found in transgenic plants (Fig. 3a). In water stress conditions, the biomass production of non-transgenic soybeans was reduced by half (2.56 g) compared to that under well-watered conditions (6.32 g). The biomass reduction was also observed in all transgenic soybeans under water deficiency (Fig. 3b). However, transgenic soybeans maintained a growth rate and showed obviously higher biomass than non-transgenic plants.

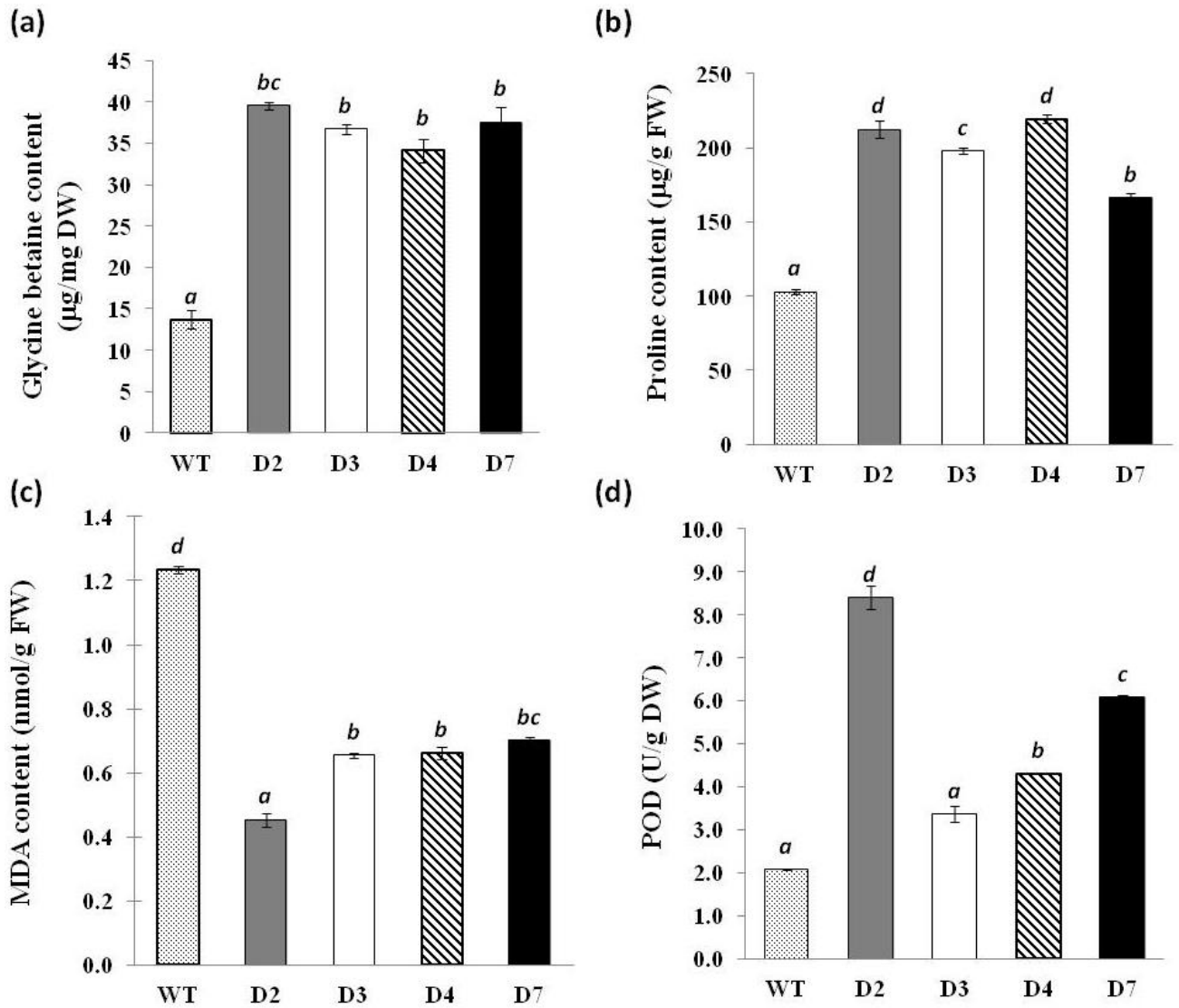

Fig. 4. Biochemical analysis of soybean leaves under drought stress. (a) Glycine betaine content. (b) Proline content. (c) MDA content. (d) POD activity. D2-D7: Transgenic soybean events; (WT) non-transgenic wild-type soybeans; Values denoted by different letters are significantly different at $p<0.05$.

Biochemical index of soybean leaves under drought stress: The increases in GB and proline accumulation were reported in several plant species under drought, salt or extreme temperatures (Bohnert *et al*., 1995; Park *et al*., 2007; Li *et al*., 2014; Zheng *et al*., 2017). To evaluate whether the inducible expression of the *codA* gene increased the accumulation of these two substances, the proline and GB contents in the soybean leaves at 9 days under stress conditions was measured (Fig. 4ab). The analysis result showed higher contents of GB and proline in all transgenic soybean leaves compared to those in non-transgenic wild-type leaves. In particular, the GB content varied from 34.1 to 39.5 μg/mg dry weight (DW) in transgenic soybeans, while the GB content for wild-type soybeans was only 13.7 μg/mg DW. The proline content was 102.9 μg/g FW in non-transgenic wild-type leaves and increased to up to 219.6 μg/g fresh weight (FW) in transgenic soybeans expressing the *codA* gene.

MDA, a chemical indicator negatively contributes to stress tolerance in plants (Hessini *et al*., 2009; Zheng *et al*., 2017). To test the oxidative damage on the membrane stability of the soybean lines, we measured the MDA contents of soybean leaves after drought stress (Fig. 4c). Our data displayed an obviously lower MDA content in all *codA* expression soybean lines than in control soybeans. Under drought conditions, the MDA content of wild-type leaves was 1.234 nmol/g FW, while the MDA content of transgenic soybeans decreased from 0.702 to 0.454 nmol/g FW. These results indicated that *codA* transgenic soybeans exhibited less severe oxidative damage than non-transgenic plants did.

The activity of POD is known as an indicator of the tolerance of soybean varieties against water stress (Zoz *et al*., 2013). Thus, we determined the activity of POD in soybean leaves after drought stress (Fig. 4d). The activities of POD were found to be higher in all transgenic soybeans than in non-transgenic ones. The transgenic line D2 exhibited the highest activity of POD (8.402 U/g DW). This result is highly consistent with the MDA data, in which the lowest MDA content was observed in transgenic line D2.

Discussion

In this study, the plant codon-optimized *codA* gene under driven by *rd29A* promoter was introduced into soybeans. PCR and Southern blot results confirmed the presence of codA in transgenic soybeans. The transgenic soybeans exhibited a faster growth rate as well as greater biomass production compared to non-transgenic soybeans at water deficit conditions. In addition, the accumulation of GB, proline and the activity of POD were consistent with previous reports for the overexpression of the *codA* gene in some plant species (Park *et al*., 2004; Ahmad *et al*., 2008; Cheng *et al*., 2013).

Of various GB biosynthetic genes, the *codA* gene encoding for choline oxidase has a function in conversion choline into betaine in the GB biosynthesis pathway (Deshnium *et al*., 1995). The transgenic plant of several species expressed the *codA* gene showed an improved tolerance to various abiotic stresses. For example, *codA*-transgenic Arabidopsis showed GB accumulation in chloroplasts and significant freezing tolerance (Sakamoto *et al*., 2000). The increased GB accumulation in transgenic tomato plants protected reproductive organs under chilling stress (Park *et al*., 2004). In our study, GB accumulation in *codA*-transgenic soybeans was higher than in non-transgenic soybeans under drought stress. Higher GB contents may reduce the water loss of transgenic soybeans and maintain vegetative growth leading to the higher biomass production of transgenic soybean plants. The *codA*-transgenic potatoes also performed similar responses under drought conditions (Ahmad *et al*., 2008). Moreover, the improved salt tolerance were found in the transgenic tobacco or potatoes and alfalfa overexpressing the *codA* gene under controlled by the stress-inducible *rd29A* or SWPA2 promoter, respectively (Jingjiang *et al*., 2013; Ahmad *et al*., 2008; Li *et al*., 2014). Therefore, further research needs to be conducted to evaluate the responses of *codA*-transgenic soybeans to other environmental stress conditions.

In addition to glycine betaine, free proline was also accumulated in plants under environmental stresses (Hayat *et al*., 2012). The overexpression of genes related to the proline biosynthesis pathway in transgenic plants exhibited higher free proline content as well as higher drought stress tolerance (Yamada *et al*., 2005; Gubiš *et al*., 2007; Zheng *et al*., 2017). Moreover, the increase in free proline content was also found in ectopic *codA*-transgenic alfalfa during a water deficit (Li *et al*., 2014). Consistently, in our study, ectopic *codA*-transgenic soybeans showed increased free proline content as well as GB accumulation under water limitation conditions. At 9 days after drought treatment, the free proline content in transgenic soybeans was 1.5- to 2 fold increase compared to non-transformed soybean plants. Similarly, the free proline content increased by up 2-fold in transgenic alfalfa with *codA* or *EDT1* under drought conditions (Li *et al*., 2014; Zheng *et al*., 2017). However, the proline content increased by less than 20% in transgenic soybeans that overexpressed the betaine aldehyde dehydrogenase (*BADH*) gene (Qin *et al*., 2017). Again, our study supported the importance of the *codA* gene in the drought tolerance of soybeans.

The increased MDA, an unsaturated fatty acid peroxidation production was considered as an indicator of cell membrane damage (Hessini *et al*., 2009). In addition to MDA content, the POD activity is known as an index of tolerance of soybean genotypes against water deficiency. The increase in POD activity was related to the greater tolerance of soybean varieties to drought conditions (Zoz *et al*., 2013). In this study, ectopic *codA*-transgenic soybeans exhibited obviously lower MDA contents and higher POD activity than wild-type soybeans under drought conditions, indicating less oxidative damage to the cell membranes of transgenic plants. In *BADH*-transgenic soybeans, MDA content decreased by as much as 13% compared to non-transgenic wild-type soybeans under drought conditions; in contrast, POD activity increased up to 7% (Qin *et al*., 2017). In this study, POD activity of transgenic plants increased up to 4-fold compared to non-transgenic control soybeans, while MDA decreased by half. The greater changes in MDA and POD may be due to the roles of the *codA* gene and rd29A promoter in transgenic soybeans.

In conclusion, we successfully introduced the *codA* gene regulated by the rd29A promoter into soybeans using the *Agrobacterium*-mediated method. Transgenic soybeans showed the faster growth rate and improved biomass production compared to non-trasgenic soybeans under drought conditions. The drought stress tolerance of *codA*-transgenic soybeans resulted from the increase in glycine betaine accumulation as well as higher proline content. Our study highlights the potential for utilizing the *codA* gene and rd29A promoter to improve drought tolerance in soybeans and other important crops.

Acknowledgments

We are grateful to Dr. Tran Thi Truong, Legumes Research and Development Center, Vietnam Academy of Agricultural Sciences for providing DT22 seeds. We want to thank Dr. Zhanyuan J. Zhang (University of Missouri-Columbia, USA) and Dr. Van Tuong Nguyen (Vietnam Academy of Science and Technology) for proofreading this manuscript. This research was funded by Vietnam Academy of Science and Technology (VAST) through Project: VAST02.02/16-17, in Scientific Program: Biotechnology, code: VAST02.

References

Ahmad, R., M.D. Kim, K.H. Back, H.S. Kim, H.S. Lee, S.Y. Kwon, N. Murata, W.I. Chung and S.S. Kwak. 2008. Stress-induced expression of choline oxidase in potato plant chloroplasts confers enhanced tolerance to oxidative, salt, and drought stresses. *Plant Cell Rep.*, 27(4): 687-698.

Alia, H., H. Hayashi, T.H.H. Chen and N. Murata. 1998b. Transformation with a gene for choline oxidase enhances the cold tolerance of *Arabidopsis* during germination and early growth. *Plant Cell Environ.*, 21: 232–239.

Alia, H. Hayashi, A. Sakamoto and N. Murata. 1998. Enhancement of the tolerance of *Arabidopsis* to high temperatures by genetic engineering of the synthesis of glycinebetaine. *Plant J.*, 16(2): 155-161.

Amanda, de P.R.A., Josirley de FCC, R. Fuganti-Pagliarini, C. Engels, A. do Rio, S.R.R. Marin, M.C.N. Oliveira de, M.A. Beneventi, F.C. Marcelino-Guimarães and J.R.B. Farias.

2014. Phenotyping soybean plants transformed with rd29A:AtDREB1A for drought tolerance in the greenhouse and field. *Transgenic Res.*, 23(1): 75-87.

Bohnert, H.J., D.E. Nelson and R.G. Jensen. 1995. Adaptations to Environmental Stresses. *Plant Cell.*, 7(7): 1099-1111.

Bradford, M.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, T.H.H. and N. Murata. 2008. Glycinebetaine: an effective protectant against abiotic stress in plants. *Trends Plant Sci.*, 13(9): 499-505.

Chen, T. and B. Zhang. 2016. Measurements of proline and malondialdehyde content and antioxidant enzyme activities in leaves of drought stressed cotton. *Bio-Protocol.*, 6(17): e1913. doi: 10.21769/BioProtoc.1913.

Cheng, Y.J., X.P. Deng, S.S. Kwak, W. Chen and A.E. Eneji. 2013. Enhanced tolerance of transgenic potato plants expressing choline oxidase in chloroplasts against water stress. *Bot Stud.*, 54: 30. doi: 10.1186/1999-3110-54-30.

Dellaporta, S.L., J. Wood and J.B. Hicks. 1983. A plant DNA minipreparation: version II. *Plant Mol Bio. Report.*, 1(4): 19-21.

Deshnium, P., D.A. Los, H. Hayashi, L. Mustardy and N. Murata. 1995. Transformation of Synechococcus with a gene for choline oxidase enhances tolerance to salt stress. *Plant Mol. Biol.*, 29(5): 897-907.

Di, R., V. Purcell, G.B. Collins and S.A. Ghabrial. 1996. Production of transgenic soybean lines expressing the bean pod motile virus coat protein precursor gene. *Plant Cell Rep.*, 15(10): 746-750.

Grieve, C.M. and S.R. Grattan. 1983. Rapid assay for determination of water soluble quaternary ammonium compounds. *Plant Soil.*, 70(2)*:* 303-307.

Gubiš, J., R. Vaňková, V. Červená, M. Dragúňová, M. Hudcovicová, H. Lichtnerová, T. Dokupil and Z. Jureková. 2007. Transformed tobacco plants with increased tolerance to drought. *S. Afr. J. Bot.*, 73: 505-511.

Hayashi, H., Alia, L. Mustardy, P. Deshnium, M. Ida and N. Murata. 1997. Transformation of *Arabidopsis thaliana* with the codA gene for choline oxidase; accumulation of glycinebetaine and enhanced tolerance to salt and cold stress. *Plant J.*, 12(1): 133-142.

Hayat, S., Q. Hayat, M.N. Alyemeni, A.S. Wani, J. Pichtel and A. Ahmad. 2012. Role of proline under changing environments: a review. *Plant Signal Behav.*, 7(11): 1456-1466.

Hessini, K., J.P. Martínez, M. Gandour, A. Albouchi, A. Soltani and C. Abdelly. 2009. Effect of water stress on growth, osmotic adjustment, cell wall elasticity and water-use efficiency in Spartina alterniflora. *Environ Exp Bot.*, 67: 312-319.

Jingjiang, H. Li, G. He, Y. Yin, M. Liu, B. Liu, G. Qiao, S. Lin, L. Xie and R. Zhuo. 2013. Over-expression of the codA gene by RD29a promoter improves salt tolerance in *Nicotiana tabacum*. *Pak J Bot.*, 45(3): 821-827.

Kathuria, H., J. Giri, K.N. Nataraja, N. Murata, M. Udayakumar and A.K. Tyagi. 2009. Glycinebetaine-induced water-stress tolerance in codA-expressing transgenic indica rice is associated with up-regulation of several stress responsive genes. *Plant Biotechnol. J.*, 7: 512-526.

Kim, H.J., H.S. Cho, J.H. Pak, T. Kwon, J.H. Lee, D.H. Kim, D.H. Lee, C.G. Kim and Y.S. Chung. 2018. Confirmation of drought tolerance of ectopically expressed AtABF3 gene in soybean. *Mol. Cells*, 41(5): 413-422.

Lenssen, B.A. 2012. How drought impacts soybean yields. *Prairie Farmer.*, p. 2012.

Li, C., H. Zhang, X. Wang and H. Liao. 2014. A comparison study of Agrobacterium-mediated transformation methods for root-specific promoter analysis in soybean. *Plant Cell Rep.*, 33(11): 1921-1932.

Li, Y., Q. Chen, H. Nan, X. Li, S. Lu, X. Zhao, B. Liu, C. Guo, F. Kong and D. Cao. 2017. Overexpression of GmFDL19 enhances tolerance to drought and salt stresses in soybean. *PLOS ONE.*, 12(6): e0179554. doi.org/10.1371/journal.pone.0179554.

Margie, M.P., H. Shou, Z. Guo, Z. Zhang, A.K. Banerjee and K. Wang. 2004. Assessment of conditions affecting Agrobacterium-mediated soybean transformation using the cotyledonary node explant. *Euphytica.*, 136(2): 167-179.

Murata, N., P.S. Mohanty, H. Hayashi and G.C. Papageorgiou. 1992. Glycinebetaine stabilizes the association of extrinsic proteins with the photosynthetic oxygen-evolving complex. *FEBS Letters.*, 296(2): 187-189.

Papageorgiou, G.C. and N. Murata. 1995. The unusual stron stabilizing effects of glycine betaine on the structure and function of the oxygen-evolving Photosystem II complex. *Photosynth. Res.*, 44(3): 243-252.

Park, E.J., Z. Jeknic, Pino, N. Murata and T.H.H. Chen. 2007. Glycinebetaine accumulation is more effective in chloroplasts than in the cytosol for protecting transgenic tomato plants against abiotic stress. *Plant Cell Environ.*, 30(8): 994-1005.

Park, E.J., Z. Jeknic, A. Sakamoto, J. DeNoma, R. Yuwansiri, N. Murata and T.H.H. Chen. 2004. Genetic engineering of glycinebetaine synthesis in tomato protects seeds, plants, and flowers from chilling damage. *Plant J.*, 40(4): 474-487.

Phang, T.H., G. Shao and H.M. Lam. 2008. Salt Tolerance in Soybean. *J Integr Plant Biol*, 50(10): 1196-1212.

Qin, D., C.L. Zhao, X.Y. Liu and P.W. Wang. 2017. Transgenic soybeans expressing betaine aldehyde dehydrogenase from *Atriplex canescens* show increased drought tolerance. *Plant Breed.*, 136(5): 699-709.

Sakamoto, A. and N. Murata. 2000. Genetic engineering of glycinebetaine synthesis in plants: current status and implications for enhancement of stress tolerance. *J. Exp. Bot.*, 51(342): 81-88.

Sakamoto, A., R. Valverde, Alia, T.H. Chen and N. Murata. 2000. Transformation of Arabidopsis with the codA gene for choline oxidase enhances freezing tolerance of plants. *Plant J.*, 22: 449-453.

Soystats. 2017. http://www.soystats.com/

Sulpice, R., H. Tsukaya, H. Nonaka, L. Mustardy, T.H.H. Chen and N. Murata. 2003. Enhanced formation of flowers in salt-stressed *Arabidopsis* after genetic engineering of the synthesis of glycine betaine. *Plant J.*, 36(2): 165-176.

Wright, D. and A.W. Lenssen. 2013. Staging Soybean Development. *Agriculture and Environment Extension Publications*., 191.

Yamada, M., H. Morishita, K. Urano, N. Shiozaki, K. Yamaguchi-Shinozaki, K. Shinozaki and Y. Yoshiba. 2005. Effects of free proline accumulation in petunias under drought stress. *J. Exp. Bot.*, 56(417): 1975-1981.

Zhang, Z., A. Xing, P. Staswick and T.E. Clemente. 1999. The use of glufosinate as a selective agent in Agrobacterium-mediated transformation of soybean. *Plant Cell Tiss. Org. Cult.*, 56(1): 37-46.

Zheng, G., C. Fan, S. Di, X. Wang, C. Xiang and Y. Pang. 2017. Over-Expression of *Arabidopsis* EDT1 gene confers drought tolerance in alfalfa (*Medicago sativa* L.). *Front Plant Sci.*, 8: 21-25. doi.org/10.3389/fpls.2017.02125

Zoz, T., F. Steiner, V.F. Guimarães, D.D. Castagnara, C.C. Meinerz and R. Fey. 2013. Peroxidase activity as an indicator of water deficit tolerance in soybean cultivars. *Biosci J.*, 29(5): 1664-1671.

(Received for publication 2 August 2018)