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DEHYDRIN *AmDH1* IMPROVES DROUGHT TOLERANCE IN TRANSGENIC MAIZE PLANTS

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SUMMARY

Dehydrin is a large protein group to have protective functions to plant cells under osmotic stress. Overexpression of dehydrin encoded genes isolated from mangrove plants had improved drought tolerance of transgenic plants. Therefore, in this research, a coding sequence with the length of 573bp that encodes Dehydrin1 (DH1) protein was isolated from a mangrove plant namely Mam bien (*Avicennia marina*) which naturally grows in Xuan Thuy national park, Vietnam. The coding sequence was denoted as *AmDH1* and cloned into the plant transformation vector pCAM 35S-*AmDH1*. The transgenic vector was transformed into *A. tumefiaciens* cells and the vector was transferred into the K7 maize line to generate transgenic maize seedlings. The *AmDH1* transgenic maize T2 seedlings have shown an improved drought tolerance than the original K7 maize line.

Keywords: *AmDH1 gene, Avicennia marina,* maize, osmotic stresses, transgenic plant.

INTRODUCTION

Dehydrin is a typical protein group highly expressed in osmotic stresses such as salinity and drought in plants. One of the proposed functions of dehydrins is the water potential increment of the cellular cytoplasm, thus the water loss during stress conditions is reduced (Close, 1996; Garay-Arroyo *et al*., 2000). The transgenic maizes (Zea mays) carrying endogenous Dehydrins such as *TAS14* and *RAB28* showed an improved tolerance against osmotic stresses (Amara *et al*., 2013, Munoz-Mayor *et al*., 2012). In addition, the transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* which overexpress a dehydrin of rice *OsLEA3-2* or of *Citrus unshiu CuCOR19* also showed a greater stress resistance ability (Duan, Cai, 2012; Hara *et al*., 2003).

Mangrove species such as *Avicennia marina, A. officinalis* and *Sesuvium portulacastrum* that grow in high salt concentration environments are potential targets for isolating genes specifically involved in stress tolerance such as salinity and drought. Genes encoding key enzymes involved in ascorbate biosynthesis such as monodehydroascorbate reductase *(MDAR)* and Superoxide dismutase *(SOD)* were successfully isolated from *A. marina*t. The expression level of other Dehydrins such as *AoDHN1* of *A. officinalis* and Fructose-1,6-bisphosphate aldolase *(SpFBA)* of *S. portulacastrum)* also increased significantly during artificial osmotic

stress treatments (salinity, drought, cold) (Jyothi-Prakash *et al*., 2014; Fan *et al*., 2009). The growth of *E. coli* expressing *AoDHN1* and *SpFBA* significantly improved in the high-salt culture medium, suggesting these genes may have protective functions against osmotic stress.

In this research, gene encoding dehydrin1 (DH1) protein was isolated from *A. marina* widely growing in Xuan Thuy national park – a mangrove forest of Vietnam. The *AmDH1* gene was transformed into maize and transgenic plants exhibited slightly tolerance to water stress.

MATERIALS AND METHODS

Materials

The leaves of *A. marina* collected from the mangrove area of Xuan Thuy National Park were washed and soaked in RNAlater RNA Stabilization solution (Thermo Fisher) for the subsequent RNA extraction and cDNA synthesis experiments. The pCAMBIA1300 vectors were stored at the Genome Research Institute. The K7 maize line was provided by the Maize Research Institute.

Table 1. Primers for *AmDH1 gene* isolation.

Name	Sequence (5'-3')	Gene	Amplicon size
5'DHN1	5' GGATCCATGTCAGAGTACGGCGAC 3'	Dehydrin 1	573bp
3'DHN1	5'TCTAGATTAATGGTGGCCTCCGGGCAG 3'		
35SProF1	5'CACTGACGTAAGGGATGACGC 3'	35S promoter position	
PolyAR1	5' TACAATACATACTAAGGGTTTC 3'	35S terminator position	

Methods

Isolation, cloning and sequence analysis of AmDH1

Total RNA was isolated from leaves of *A. marina* collected from the mangrove area of Xuan Thuy National Park, washed and soaked in RNAlater RNA Stabilization solution (Thermo Fisher) using Trizol reagent (Thermo Fisher). RNA concentration was determined by the NanoDrop ND-1000 UV-Vis spectrophotometer.

Two micrograms of total RNA was used for the cDNA synthesis using the SMARTER RACE cDNA synthesis kit (Clontech, CA, US). Targeted gene was amplified using RACE-PCR method with Prime STARMAX Pfu polymerase (Takara, Japan) and 5'DHN1 (5' GGATCCATGTCAGAGTACGGCGAC-3') and 3'DHN1 (5'TCTAGATTAATGG TGGCCTCCGGGCAG-3') primers. The amplified gene were cloned into pCR4Blunt-TOPO vector (Invitrogen). The recombinant vector was selected by colony-PCR with M13F/M13R primer pair and confirmed by Sanger sequencing

Construction of plant expression vector and Agrobacterium mediated transformation into maize

The plant expression vector pCAMBIA1300 35S-*AmDH1* was constructed using restriction enzyme digestion, ligation and transformation according to Sam Brook et al. (2001).

Agrobacterium tumefaciens-mediated transformation was conducted using immature embryo of K7 maize line (the Maize Research Institute) according to Frame et al (2006) with modification. Immature embryos of 1-2 mm in size were separated from 10-12 day after pollinated seeds under sterile conditions and inoculated with a bacterial solution supplemented with 100 µM AS for 20 min. Infected embryos were placed to a co-culture medium (CCM) and kept in the dark, at 21^{o}C for 3 days before transferring into recovery medium (RE) in the dark at 28^{o}C, for 7 days. Then, callus formed from immature embryos was transferred

to selection medium 1 (SE1) and selection medium 2 (SE2) supplemented with 1.5 mg/l and 3,0 mg/l hygromycin, respectively, in the dark at 28°C for 14 days each. Embryonic callus formed in SE2 medium was transferred to regeneration medium 1 (RM1) containing 3 mg/l hygromycin without plant growth regulator, kept in the dark for 7 days at 25°C. The embryogenic callus was subcultured to regeneration medium 2 (RM2) without selectable antibiotics under light condition (16h light/day) at 25°C until plants regenerated. Regenerated shoots were transplanted to a rooting medium under light conditions, 25°C, for about 10-20 days. Regenerated plants reaching three - leaf stage were transferred into the soil. The transgenic plants were confirmed using PCR analysis.

Evaluation of transgenic plant of drought tolerance at seedling stage

The drought stress at seedlings stage of transgenic plants expressing *AmDH1* was conducted in the green house. Non-transgenic K7 maize plants were used as the control. At early stages, plants were watered and fertilized sufficiently to ensure uniform growth, Water stress treatment was done with 5-7 leaf seedlings by no watering for 14 days and then water again for recovery. Plants in the control batch still irrigated normally. During the drought period, the degree of wilting of leaves were recoded. The survival rate and morphological characteristics of the plants were assessed at recovery phase. Root volume, root length, dry stem weight were evaluated according to the method of Camacho and Caraballo (1994).

RESULTS AND DISCUSSION

Cloning and sequencing *AmDH1 gene*

In this research, the open reading frame of *AmDH1* was amplified from the cDNA of the mangrove plant *A. marina* using specific primers 5'bDH1 and 3'bDH1 (Table 1), which were designed based on mRNA sequence of *A. officinalis DH1* (Genbank caccession no. KM652423). The target amplicon had a size of 0.6 kb as expected, then purified and ligated into pCR4Bunt-TOPO. The recombination vectors after transferring into *E. coli* were selected by colony-PCR using primer pair M13F and M13R. Two of *AmDH1* positive plasmids (No. 13 and 14) were sequenced. The result showed that they all had the same length of 573bp encoding 191 amino acids of DH1 protein. The sequence analysis revealed that isolated *AmDH1* of *A. marina* contained the start (ATG) and terminator (TAG) sites and 97.2% identical to the reference sequence of *A. officinalis*. There are 15 single base differences at 33, 69, 85, 151, 168, 170, 230, 260, 270, 287, 304, 321, 337, 465 and 501 positions (Figure 1). However, the differences in amino acid sequences only occur at 7 positions of 29, 51, 57, 77, 94, 102, and 113 (Figure 1). The obtained *AmDH1* sequence in this study was registered on the Genbank (accession no. KT987468).

Construct of transformation vector expressing *AmDH1*

To express the *AmDH1* gene in plant cells, the final vector pCAM35S-*Am*DH1 was developed. The *AmDH1* CDS sequence which has the size of 573bp was cut from the cloning vector pCR4Blunt-TOPO with restriction enzymes *Bam*HI and *Xba*I was ligated with the opened pCAMBIA1300 backbone which was also cut with enzymes *Bam*HI and *Xba*I. The ligated product was transformed into *E. coli* DH5α competent cells. The recombinant plasmids were checked by PCR using primer pair 35SProF1 and PolyAR1 (Table 1) which were designed based on sequence of pCAM vector, the PCR product has size about of 700 bp (lane 1 and 16, Figure 2), suggesting a gene fragment size of about 573 bp was inserted into the pCAM1300.

Together with sequencing (data not shown), we confirmed that the *AmDH1* coding sequence was successfully inserted into pCAM1300 to form the pCAM35S-*Am*DH1. The expression vector pCAM35S-*Am*DH1 contain a selective gene for kanamycin resistance in bacteria and hygromycin in plants. The *AmDH1* gene located

in the 35S Pro::DH1::35S Ter expression cassette (Fig. 3 A, B). The pCAM 35S-*Am*DH1 was mobilized into *A. tumefaciens* strain C58 by freeze-thaw method. .

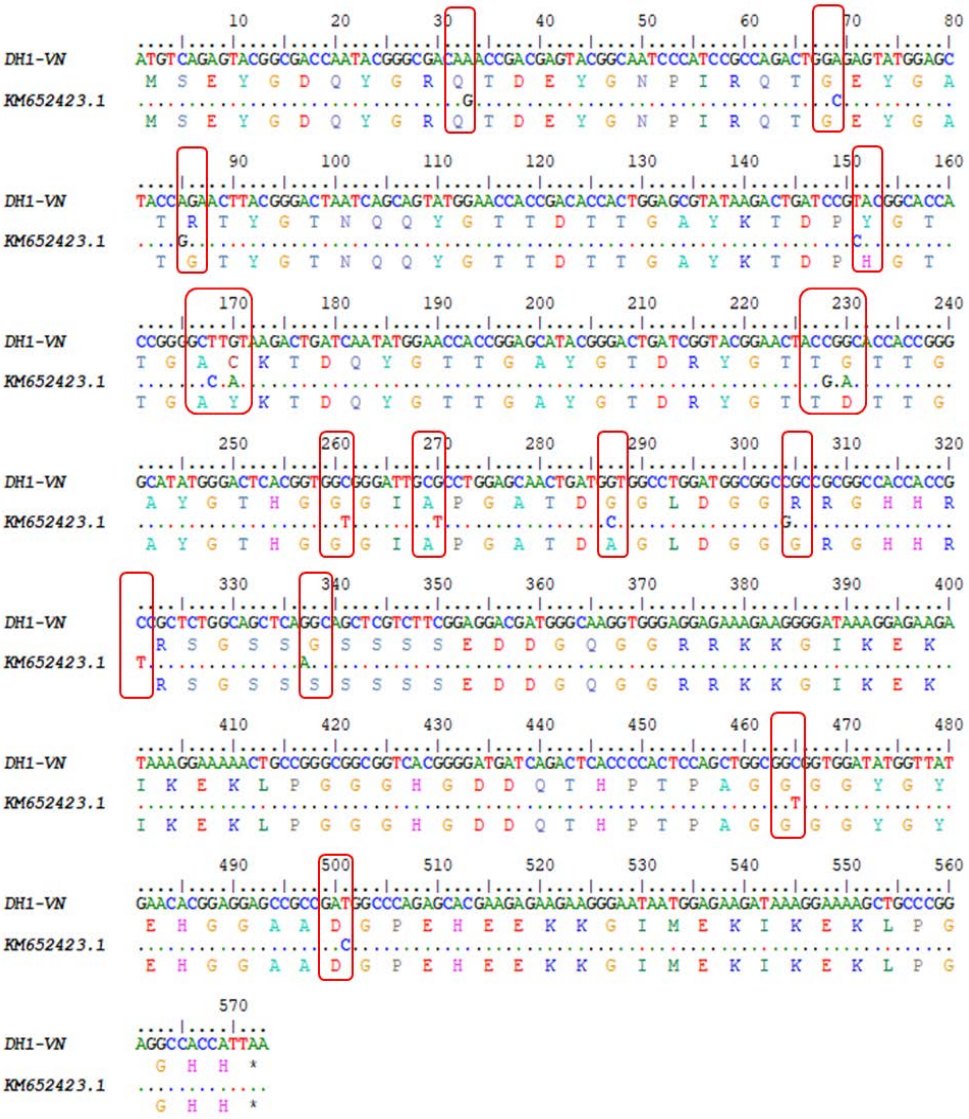

Figure 1. Sequence alignment of the isolated *AmDH1.*

Figure 2. PCR for confirming the insertion of *AmDH1* into pCAMBIA 1300. M: DNA 1kb ladder; 1-20: PCR products from relevant colonies.

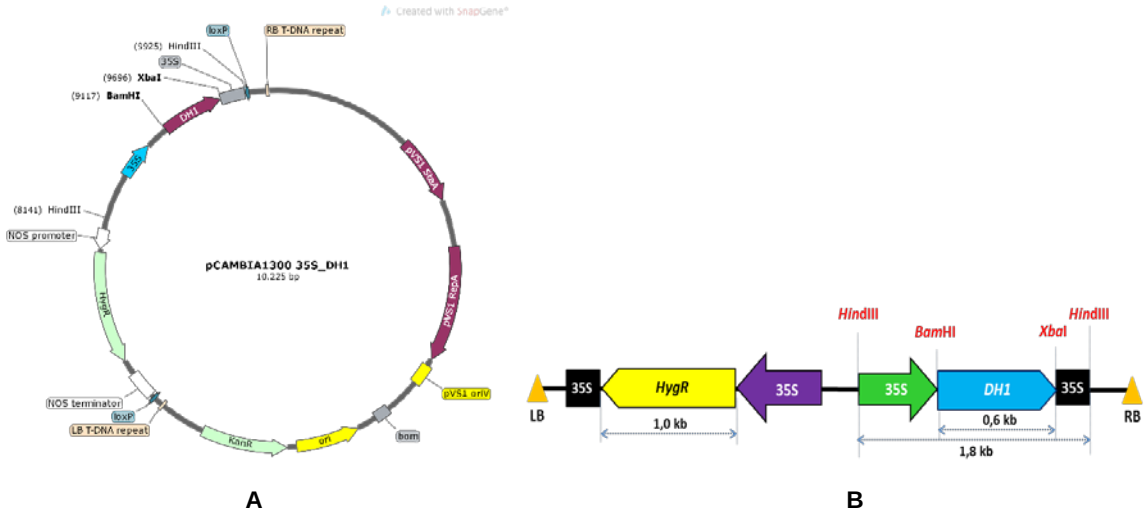

Figure 3. Vector construction map. ***A:*** *pCAM 35S-AmDH1 vector;* ***B****: 35S Pro::DH1::35S Ter cassette.*

Transformation of *AmDH1* gene into maize

In this research, plant transformation via *A. tumefaciens* C58 carrying pCAM 35S-*Am*DH1 was carried out into K7 cultivar - an elite maize using immature embryos as initial materials. The main stages of the gene transfer procedure were shown in Figures 4 and 5. After infection and co-culture with *A. tumefaciens*, the embryos were moved into the recovery medium and selection medium containing hygromycin. The number of immature embryo-derivedcalluses were decreased gradually in two selection phases due to necrosis phenomenon of non-transformed ones. The survival callus on selection medium were transferred into the regeneration medium. From 2400 immature embryos, there were 115 complete single plants regenerated, however only 88 plants were survival after two week later in green house (Table 2). All seedlings had the normal morphology until the stage of 3-5 leaves. At later stages, some seedlings had abnormal flowering phenotypes including mismatches between the time of silks emerging and tassel pollen shedding. Although seedlings had some morphological abnormalities of the outer husk, we still obtained about 10 seedlings from the T0 generation that produce seeds and their leaf samples were collected for analyzing the presence of *AmDH1* gene by PCR.

Evaluation of *AmDH1* transgenic plants

Transformation efficiency was evaluated based on the presence of the transgene in T0 transgenic seedlings. Twelve T0 plants (including 10 seed produced plants and 2 non seed produced) were selected for total DNA extraction. The presence of *AmDH1* transgenes was check by PCR using isolated DNA and the primer pair 5'bDH1 and 3'bDH1. All of them gave bands of 573bp for the *AmDH1* gene (Figure 6A). Although the PCR product is not really specific due to some unspecific bands, the main PCR products have a correct size to the positive control suggesting that *AmDH1* were inserted in the T0 seedling genome. Next, we also checked the presence of *AmDH1* genes in T1 plants of 57 and 60 T0 lines. There were two positive T1 plants for each lines 57 (57.43; 57.44) and 60 (60.61; 60.62) (Figure 6B).

The rate of successful gene transfer into maize is not high due to the difficulty of gene transfer into monocotyledonous plants (Shood *et al.*, 2011). Although the mechanism of T-DNA translocation of bacteria *A. tumefaciens* into these dicot and monocot plants was confirmed to be the same, the dicots showed a better rate of transgene acquired. The difference in gene transfer efficiency may cause by the divergence in many aspects such as cell wall structure composition, differentiation

ability, response to injury, and vir gene induction process between monocot and dicot. In addition, several secondary metabolites that inhibit the induction of vir genes required for foreign gene incorporation have been found in maize (Liu *et al*., 2006; Maresh *et al*., 2006).

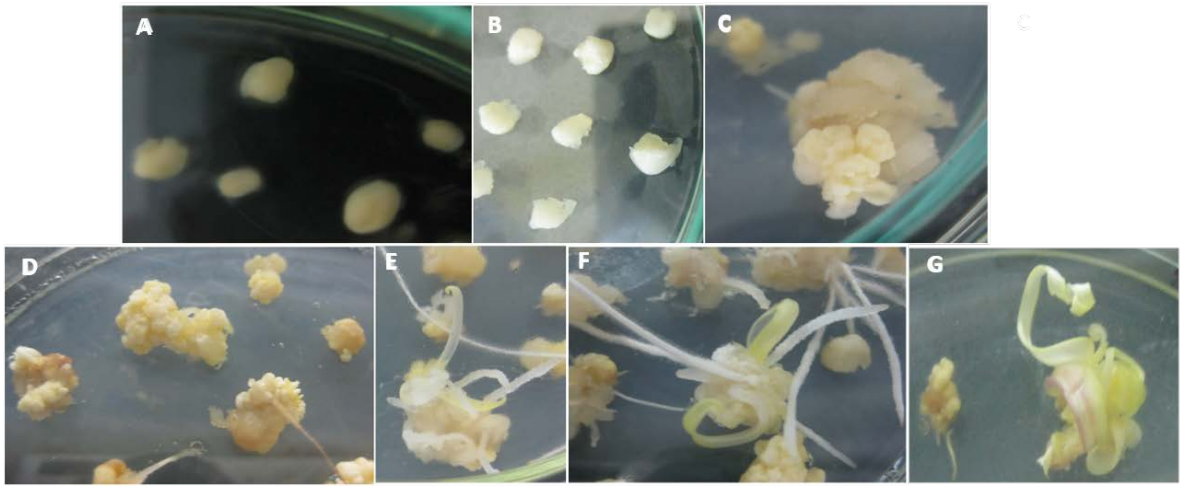

Figure 4. Transformation to immature embryos through *A. tumefaciens.* Co-culture embryos on CCM (**B**) Callus formation on RE medium. (**C-D**) Transgenic callus seletion on SE medium. (**E-G**) Plants regenerated on RM1.

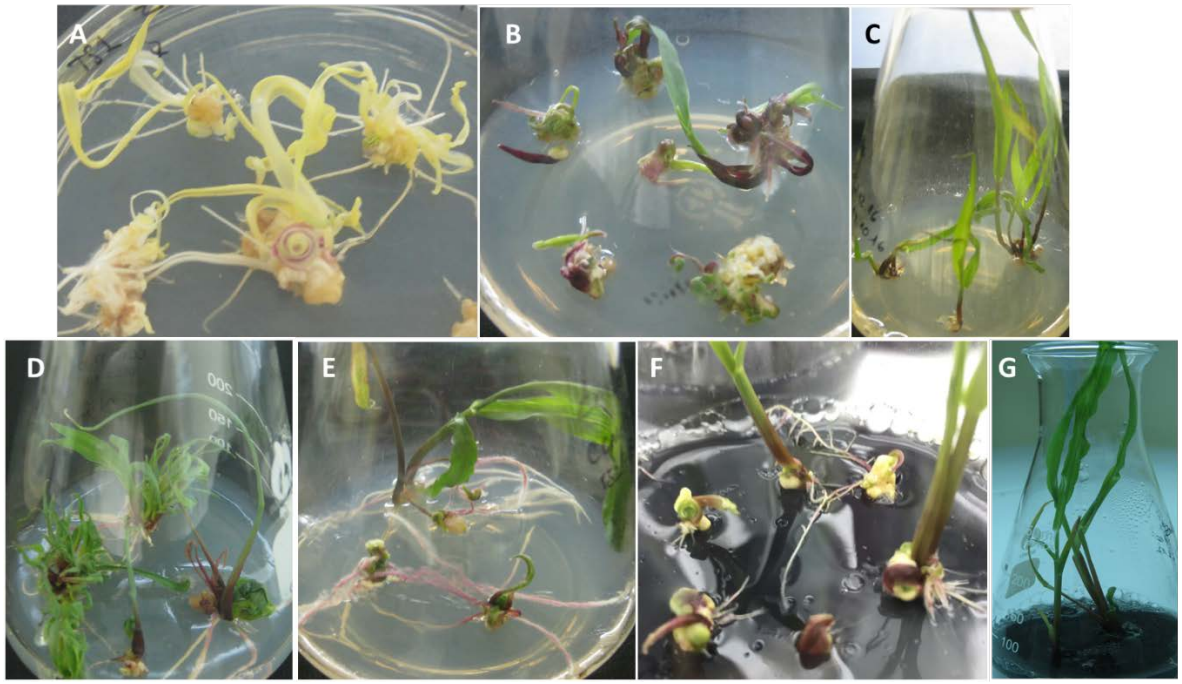

Figure 5. Transgenic maize plants regenerated on selection medium. (**A-E**) Plants regenerated on RM2. (**F-G**) Plants rooted on RR.

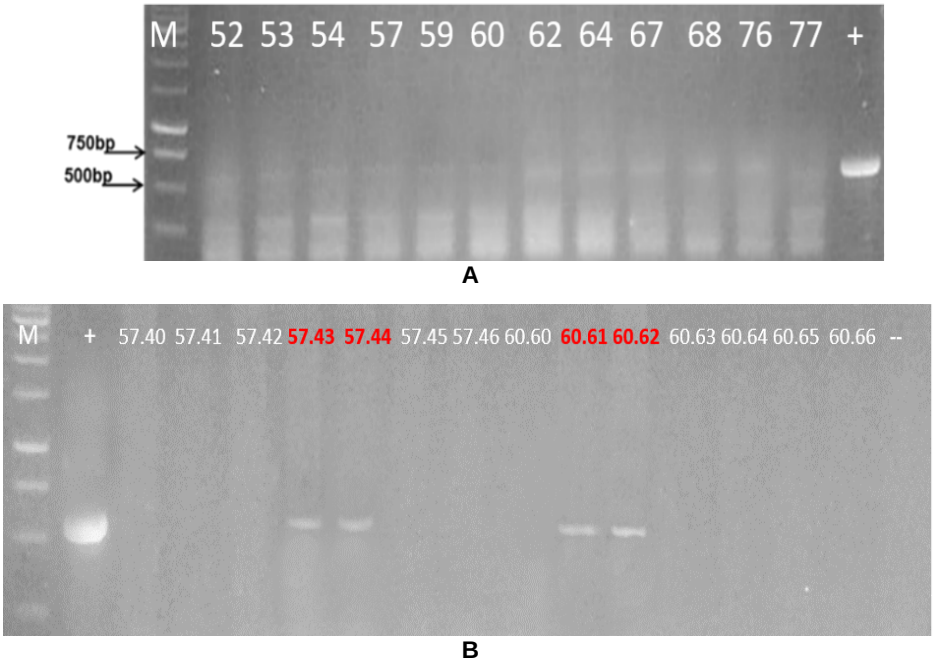

Figure 6. PCR analysis of transgenic maize plants. **A**. Transgenic plants of the T0 generation; **B**. T1 generation transgenic plants; M: DNA ladder; (+): positive control with template was plasmid pCAM 35S-*Am*DH1, (-): negative control with water template.

Evaluation of drought tolerance at seedling stage with *AmDH1* transgenic plants

Seedlings of transgenic plants *AmDH1* derived from the 57.43 plant and seedlings of K7 control plants were observed under three conditions: watering; artificial drought stress; and recovery after re-watering. Germination and growth performance under normal watering and fertilization conditions showed no significant difference between the transgenic plants and the K7 lines (Figure 7A). In the drought treatments, we stopped watering for 14 days. The leaf curl of both transgene plants and K7 plants was at a high level. Particularly in the K7, maize leaves showed the early senescence phenotype (Figure 7B). At to the recovery stage, there was a clear difference between these two plots. The leaves of the *AmDH1* transgenic plants turned into greener, fresher more rapidly than that of the K7 line (Figure 9C). Moreover, the roots of transgenic plants grew longer (number 2 figure 7D), with more branches than that of the K7 line suggesting better water absorption ability during drought conditions. (Figure 7 D).

Along with the morphological difference between the *AmDH1* transgenic plants and K7 lines after recovery, the survival rate of the transgenic plants (86.0%) was more than 2 times higher than that of the K7 line (40.0%). In addition, some growth parameters such as length, volume/weight of root, fresh stem, leaf dry stem, and total dry biomass of the plants were recorded (Table 2).

Under normal conditions, the parameters observed between the *AmDH1* transgenic maize and the K7 line are similar with no statistically significant difference with 95% confidence. However, in artificial drought treatment, the *AmDH1* transgenic plants showed superiority over the K7 line. Total dry biomass under drought conditions of the *AmDH1* transgenic plants was 1.96 g or 60.2% significantly higher than that of the K7 (0.78g) with 95% confidence. The dry weight reflects the growth and development of the plant. The plant grows and

develops better, the greater the amount of dry matter accumulated in the plant. In addition, the indices of leaf length, root length, root volume, fresh leaf weight, fresh root weight, dry root weight, and dry stem weight of the *AmDH1* transgenic plants were all higher than those of the K7 line. These indicate that transgenic maize plants expressing *AmDH1* have better tolerance than the original K7 line under drought conditions.

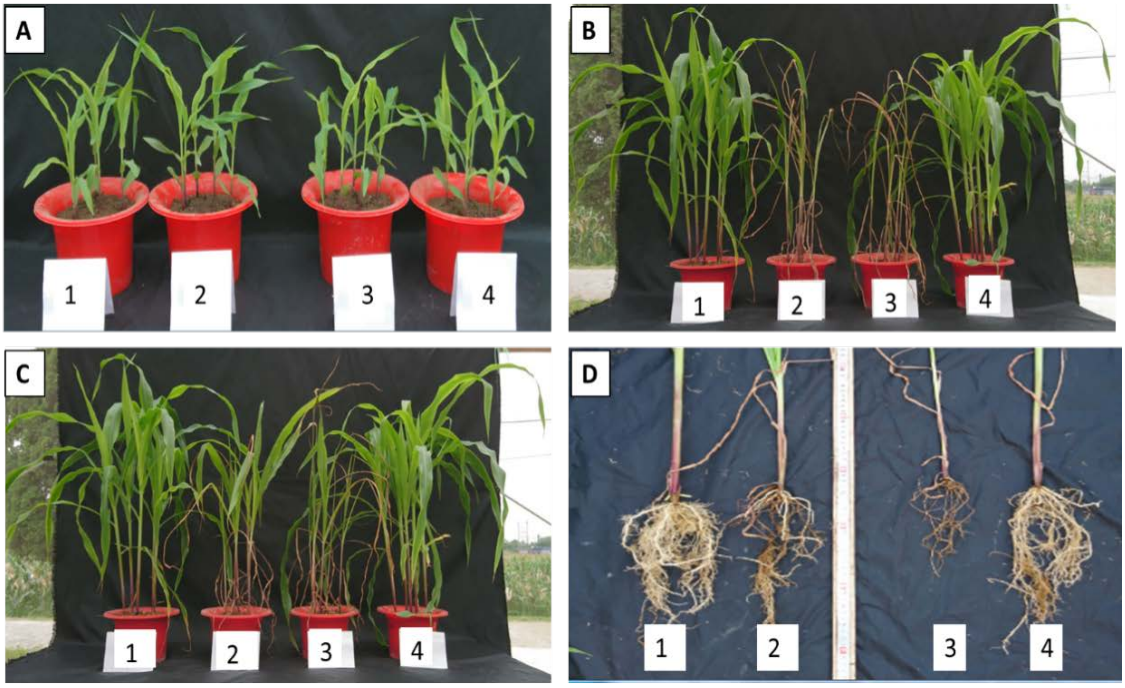

Figure 7. Drought treatment of T2 transgenic *AmDH1* seedlings in a greenhouse. **A.** Maize seedling at the 5-leaf stage; **B.** Maize seedlings after 14 days of drought treatment; **C.** Maize seedlings recovered after 7 days (number 2, 3); **D.** Roots of maize plants after recovery 1, 2: *AmDH1* transgenic plants in normal conditions and drought stress; 3, 4: K7 plants in drought stress and normal conditions.

Table 2. Growth and development of *AmDH1* transgenic maize.

Batch	Conditions	Leaf length (cm)	Root length (cm)	Root volume (ml)	Stem fresh weight (g)	Root fresh weight (g)	Dry Stem weight (g)	Dry root weight (g)	Dry biomass weight (g)
AmDH1 transgenic plant	Normal	99.0	27.7	9,1	23,16	10,32	3,57	1,51	5,08
	Drought stress	80.0	20,2	4,2	7,27	4,43	1,37	0,59	1,96
K7 line	Normal	98.34	26,8	8,7	22,35	10,01	3,48	1,47	4,95
	Drought stress	68,5	14,8	2,4	4,28	2,19	0,64	0,14	0,78
CV%		6,0	5,6	7,9	4,4	8,3	8,0	5,8	6,7
LSD 0,05		10,81	2,53	0,98	1,17	1,05	0,34	0,10	0,40

CONCLUSIONS

In this study, the *AmDH1* gene from *A. marina* was successfully isolated and sequenced with 573 nucleotides in length. The *AmDH1* open reading frame was transferred into the K7 maize line through *A. tumefaciens* transformation. The T0 and T1 *AmDH1* transgenic plants were confirmed by PCR. The T2 seedling stage showed the improved drought tolerance in comparison with the original K7 line.

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