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Overexpression of a soybean expansin gene, *GmEXP1*, improves drought tolerance in transgenic tobacco

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Abstract: The *EXP1* gene encodes expansin, which has the ability to loosen the plant cell wall. The soybean expansin gene *GmEXP1* is activated specifically during the root elongation process, and thus it plays important roles in root development. During the drought period, changes in pressure within the cell and the fast development of the root allow plants to collect water from deep soil, which in turn helps plants grow and develop. In this study, we have successfully cloned and generated a *GmEXP1* construct expressing recombinant expansin protein in tobacco plants. *GmEXP1* is expressed in transgenic tobacco plants and passed on to the next generation. The transgenic tobacco plants have improved drought tolerance, which is demonstrated in both the length and volume of roots. From these promising results, we applied the same approach to generate drought-tolerant plants.

Key words: *Agrobacterium*-mediated transformation, expansin, *GmEXP1* gene, loosening of cell wall, root elongation, soybean

1. Introduction

Drought stress is one of the most important yield-reducing factors in crop production. The molecular basis of drought tolerance mechanisms in plants have attracted interest: one study aimed to identify the protein profiles and dehydrin accumulation in seven varieties of local Indonesian soybeans (Arumingtyas et al., 2013). The root is an important part of the plant. It is the main water absorber and distributer to cells so that it can be distributed throughout the plant. During drought periods, plants with long, spread out roots can collect water and nutrients and thus have a better chance to survive (Huck et al., 1983). The development of plant roots depends on genes that control morphological characteristics, growth, development, metabolism, and other physiological processes (Taylor et al., 1978; Wang et al., 2010; Uga et al., 2013; Xu et al., 2014). In a study by Makbul et al. (2011), anatomical changes in the root, stem, and leaf of soybean (*Glycine max*) plants under drought stress were studied by light microscope and their significance was evaluated by numerical analysis. In soybean, expansin protein is found at the growth domain of the main root and side roots. Expansins are required for root elongation (McQueen-Mason et al., 1994; Choi et al., 2003; Lee et al., 2003).

All 4 groups of expansin, α-expansin (EXPA), β-expansin (EXPB), expansin-like A (EXLA), and expansin-like B (EXLB), have the ability to loosen plant cell walls. These proteins are encoded by a multigene family and are found in many plants such as rice, wheat, soybean, corn, and potatoes (Kende et al., 2004). In soybean, there are 75 different *EXP* genes found on 18 chromosomes. The *GmEXP1* from chromosome 17 is 1491 nucleotides long, containing 3 exons and 2 introns. The coding region is 768 nucleotides in length, encoding α-expansin with 255 amino acids (Kende et al., 2004; Zhu et al., 2014), and consists of three regions: a signaling region, an active region similar to endoglucanase, and the pollen allergen region for substrate adhesion (Wu et al., 2001). When the cell wall reaches a pH growth level of 4.5–6.0 and the ratio of expansin and cell wall (on a dry mass basis) is 1:10,000, the expansin will cause cellular structural changes by sticking, breaking links between polysaccharide components (including pectin and hemicellulose), and loosening the polymer network. Pressure from the swelling of the cell increases the distance between the microfibers in both horizontal and vertical directions (Cosgrove, 2000, 2005). In addition to the direct impact on the cell wall, expansin also indirectly creates room for cellulase enzyme to come into contact with the substrate, effectively accelerating the increase in cell size. Some experiments have shown that the activity and distribution of expansin in soybean root

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cells play an important role in root elongation, enabling plants to absorb more water and nutrients from deeper soil layers (Cosgrove et al., 1998).

The *GmEXPB2* gene from the β-expansin group was shown to be activated under certain adverse conditions, related to root elongation, increased phosphorus absorption, and osmotic pressure changes. These are changes that are observed in response to drought condition (Guo et al., 2011, Zhou et al., 2014). In 2003, Lee et al. demonstrated the role of *GmEXP1* in cell wall expansion at the developing site of the tobacco plant root. The technology used to transform *A. tumefaciens* has opened up the possibility to create transgenic plants carrying the *GmEXP1* gene to improve root elongation for drought resistance. This work studying the expression of the *GmEXP1* gene isolated from soybean cultivars aims at improving drought tolerance in transgenic tobacco.

2. Materials and methods

2.1. Materials

Nine Vietnam local soybean cultivars, labeled SL1, Sl2, SL3, SL4, SL5, SL6, BK, VP, and DT84, were used for isolation and molecular cloning of the *GmEXP1* gene. Among these cultivars, the local cultivar SL1, which has the most developed root system, was used as the source of *GmEXP1* for transformation. The *Nicotiana tabacum* K326 tobacco plant was used to check activity of the recombinant vector. The DH5α *E. coli* strain, *A. tumefaciens* CV58, and some other vectors such as pBT, pRTRA7/3, and pCB301 were used for cloning, vector design, and transformation.

The primers used for PCR and real time RT-PCR are shown in Table 1.

2.2. Isolation, cloning, and analysis of the *GmEXP1* gene

Total RNA was isolated from soybean leaves using TRIzol Reagent (Life Technologies). cDNA was synthesized using a Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). The *GmEXP1* gene was cloned from cDNA using the Soy*EXP1*-F-NcoI and Soy*EXP1*-R-NotI primers, then ligated into the pBT cloning vector, transformed, and expressed in *E.coli* DH5α. Colony PCR was used for screening. The gene was then sequenced and investigated using BioEdit and DNASTAR software.

2.3. *Agrobacterium*-mediated transformation

Agrobacterium-mediated transformation via leaf infection and regeneration of tobacco plants was done as previously described by Topping (1998).

2.4. Real-time RT-PCR for *GmEXP1* gene expression analysis in tobacco

The transcription level of *GmEXP1* was determined by real-time RT-PCR using SYBR Green I fluorescent dye from Roche. The process was carried out as follow: 1) Extract total RNA using TRIzol Reagent (Life Technologies). 2) Synthesize cDNA using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). 3) Perform real-time RT-PCR using Roche LightCycler 480; thermal cycle: denaturation at 95 °C for 10 min; amplification and binding for 45 cycles (95 °C for 10 s, 58 °C for 10 s, 72 °C for 20 s); analyze the flow temperature when the temperature increases from 65–95 °C for 1 min and continue to collect fluorescent signals. 4) Calculate and determine expression (R) using the $R = 2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (2001). Results are presented relative to those of genes encoding actin.

2.5. Analysis of recombinant expansin protein by western blot

To extract total protein, 0.5 g of leaves were crushed in liquid nitrogen and dissolved in 1 mL of PBS with 0.05% Tween 20 (PBS-T), then centrifuged at 13000 rpm for 15 min. Proteins were denatured and run on 10% SDS-PAGE, then transferred to nitrocellulose membranes using a Pierce G2 Fast Blotter (25 V, 1.3 mA for 20 min). Membranes were then blocked in blocking solution (5% skim milk in PBS-T) overnight and incubated with primary antibody (c-myc) for 3 h by shaking at room temperature, followed by 3 washes with PBS, and then incubated with secondary antibody for 2 h. Mouse monoclonal antibody

Table 1. The primers used for PCR and real-time RT-PCR.

Primers	Nucleotide sequence (5' - 3')	Size (bp)
Soy*EXP1*F*Nco*I	CATGCCATGGATGGGCAAAATCATGCTTGT	790 (cDNA) 1513 (DNA)
Soy*EXP1*R*Not*I	ATTTGCGGCCGCTTAGAACTGAACTGGGCTAGA	
qEXP1-F	CCTATGCCTTCTCACCTTCTG	133
qEXP1-R	GCAGTTCTAGTTCCATACCCAG	
qAct-F	GATCTTGCTGGTCGTGATCTT	152
qAct-R	GTCTCCAACTCTTGCTCATAGTC	

to c-myc (Santa Cruz Biotech) was diluted in 5% milk in PBS at 1:700. For secondary antibody, antimouse IgG antibody attached HRP (horse radish peroxidase) was diluted in 5% milk in PBS at 1:4000. Results were displayed using TMB (3,3',5,5'-tetramethyl benzidine) or DAB (3,3'-diaminobenzidine tetrahydrochloride).

2.6. Evaluation of the development of transgenic roots

Seeds from transgenic plants were sown on MS medium with kanamycin (50 mg/L). After forming 2 leaves, the plants were grown on pots of yellow sand to test for drought resistance. Estimation of the drought tolerance was initiated when plants had 3 or 4 true leaves; watering was stopped in the experimental plots while it was continued in the control plots, as described by Le et al. (1998). The root length, volume, and dry mass were measured after 3, 5, 7, and 9 drought days.

3. Results

3.1. Cloning of the *GmEXP1* gene from Vietnamese soybean cultivars

The *GmEXP1* coding sequences isolated from the cDNA of 9 soybean cultivars are 99.3%–99.7% identical to sequences from GenBank (AF516879). They are 768 bp in length, encoding 255 amino acids. When compared with the classification system proposed by Zhu et al. (2014), *GmEXP1* is identical to *GmEXPA37*, an α-expansin subfamily, which is located on chromosome 17. This result is consistent with the soybean mRNA genetic map constructed by Libault et al. (2010). The *GmEXP1* cDNA sequences from soybeans were accepted by GenBank and published under the following codes: HG799004, HG799005, HG799006, HG799007, HG799008, HG799009, HG799010, LN681352, and LN681353, and the two *GmEXP1* DNA sequences are LM651915 and LM651916.

3.2. Generation and analysis of transgenic tobacco plants

3.2.1. Generation of transgenic tobacco plants

Of all the collected soybean cultivars, the cultivar SL1 from Phu Yen, Son La, has the longest root system, and thus we used it as the source for the *GmEXP1* gene. The *GmEXP1* gene (NCBI ID: HG799004) was isolated from cDNA and cloned into the pCB301 plant transgenic vector. The modified gene was designed to have the following components between the left border (LB) and right border (RB) for plant gene transfer: promoter 35S, c-myc tag, polyA, and *nptII* for kanamycin screening. The recombinant vector was transformed into tobacco plant K326 using *A. tumefaciens* CV58. PCR of *GmEXP1* in the T_0 generation leaves revealed that 32 of 44 transgenic tobacco lines carried this transgene.

From these 32 PCR-positive transgenic tobacco lines, we determined the *GmEXP1* gene activity using RT-PCR. This technique allows rapid detection of transgene activity based on mRNA production. The total RNA was extracted from leaves and was converted to cDNA using reverse transcriptase enzyme and then run on PCR using specific primers (Soy*EXP1*F/Soy*EXP1*R). The RT-PCR products were checked on a 0.8% agarose gel (Figure 1). Specific bands of approximately 0.79 kb in size appeared on lanes 26, 30, and 36 (correspond to transgenic lines 26, 30, and 36, respectively), suggesting that the *GmEXP1* gene had been successfully incorporated into the tobacco genome and transcribed into mRNA in 3/32 transgenic lines. We then used these 3 transgenic lines for further analysis.

3.2.2. Germination and selection of T_1 transgenic tobacco plants

T_0 seeds were sowed on MS medium supplemented with kanamycin for selection. We expected that the germinated and developed seeds would carry the recombinant *GmEXP1* construct. The wild-type seeds were unable to grow in kanamycin medium (Figure 2). Transgenic tobacco line number 36 did not germinate in either the control or kanamycin medium. In contrast, the seeds of

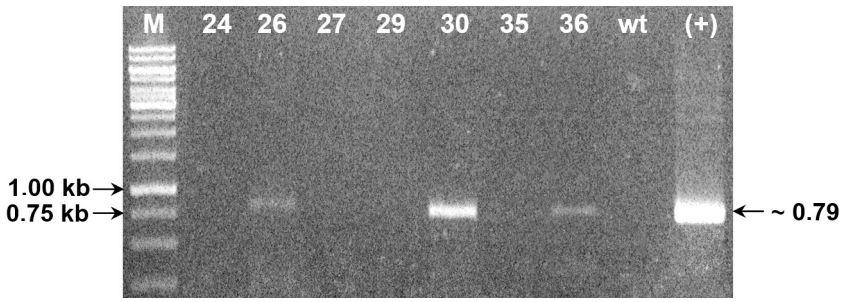

Figure 1. Electrophoresis of RT-PCR products to determine *GmEXP1* activity from transgenic tobacco lines. M: DNA ladder, 1 kb; 24–36: RT-PCR products from transgenic tobacco lines; (+): PCR product of pCB301_GmEXP1 vector; wt**:** RT-PCR product from control tobacco plants.

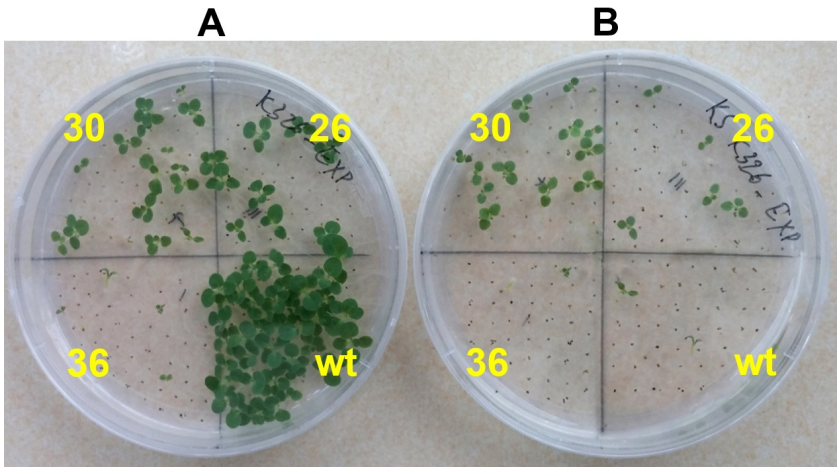

Figure 2. Tobacco seeds sown on the MS medium without antibiotic (A) and with kanamycin (B). 26, 30, 36: seeds of transgenic tobacco lines; wt: wild-type tobacco K326. Vertical bars represent standard error.

transgenic lines 26 and 30 were able to germinate and develop normally in both control and kanamycin media, indicating the proper function of the *ntpII* gene.

3.2.3. Transcription levels of the *GmEXP1* gene

To assess the transcription level of the transgene *GmEXP1* in transgenic tobacco plants, we performed real-time RT-PCR using primer pair q*EXP1*-F/q*EXP1*-R (Table 1). Actin expression was used for reference with primer pair qAct-F/qAct-R (Table 1). Results were evaluated using the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (2001).

3.2.3.1. Transcription levels of *GmEXP1* in T_0 generation transgenic tobacco plants

The transgenic tobacco plants $T_0$26, $T_0$30, and $T_0$36 were subjected to total RNA extraction and cDNA synthesis (1 µg/reaction). Real-time RT-PCR reactions allowed determination of the differences in mRNA copy number of the *GmEXP1* gene and thus displayed different levels of expression in each sample.

The graph shows the threshold cycles (Ct) through the actin and *GmEXP1* cDNA amplification at cycles 24 and 20–22, respectively. The graph of the melting temperature and peak flow shows that the real-time RT-PCR reaction amplified two main products (actin and *GmEXP1*) and no other products. Among these 3 transgenic plants, $T_0$26 has the lowest expression level and $T_0$30 has the highest. Table 2 shows the relative expression levels of *GmEXP1* in T_0 generation plants compared to the level in $T_0$26.

3.2.3.2. Transcription levels of *GmEXP1* in T_1 generation transgenic tobacco plants

Similar to the T_0 generation, real-time RT-PCR in T_1 transgenic tobacco plants displayed the same cDNA amplification curves between the control actin and *GmEXP1*. The graph also shows the melting temperature and flow peak at 82 °C (for *GmEXP1*) and 84 °C (for actin). This confirmed the existence of two amplified DNA products and no other unspecific products.

The total Ct values obtained from real-time reaction were used to identify the ΔCt values, ΔΔCt, and R expression level to compare to those of $T_0$26 using the Livak method. T_1 plants 26-1, 26-2, 26-3, 30-1, and 30-2 had similar expression levels to the $T_0$26 plants (Figure 3). When applying the statistical analysis t-test on expression level of transgenic plants belonging to $T_1$26 and $T_1$30 lines with $\alpha = 0.001$, the results of the analysis showed that $t_{stat} = 0.077$, smaller than the value t_α one-sided (10.21) and t_α two-sided (12.92). This shows that the two transgenic tobacco lines 26 and 30 have the same relative expression levels of the *GmEXP1* gene at 99.9% reliability.

Table 2. Relative *GmEXP1* gene expression in transgenic tobacco plants T_0 generation.

Samples	Ct_*EXP1*	Ct_Actin	ΔCt(T)	ΔCt(C)	ΔΔCt	R/26
$T_0$36	21.75	23.90	−2.15		−0.14	1.11
$T_0$30	20.89	23.93	−3.04		−1.03	2.04
$T_0$26	21.96	23.97		−2.01	0.00	1.00

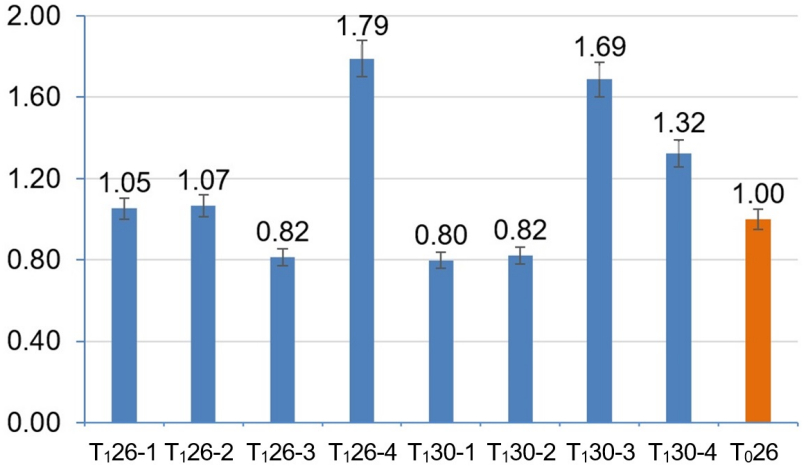

Figure 3. Relative expression level of *GmEXP1* gene in T_1 generation plants, compared to the expression level in $T_0$26. Vertical bars represent standard error.

Several plants, such as 26-4, 30-3, and 30-4, had 1.32 to 1.79 times higher transgene expression level than the $T_0$26 plant. This is due to the variation in *GmEXP1* expression from different plants, or influenced by other intracellular factors such as the position of the integrated gene on the chromosome, change in gene copy numbers through sexual reproduction, or change in expression efficiency of the target gene in later generations.

3.2.4. Analysis of recombinant expansin protein in transgenic tobacco plants

The *GmEXP1* gene is tagged with c-myc at the 3'-terminus, allowing us to determine the expression of recombinant protein using c-myc antibody. Using ExPASy tools, we predicted that the protein size is about 30 kDa. Indeed, we detected specific bands with the predicted size in the 3 T_0 generation transgenic plants (Figure 4A), suggesting that the recombinant *GmEXP1* gene had been successfully expressed in tobacco plants.

Similarly, we examined the expression of recombinant protein in T_1 generation plant lines 26 and 30, and also obtained specific bands as expected (Figure 4B). This result indicates that the *GmEXP1* transgene passed from the T_0 to T_1 generation and still expressed the recombinant expansin protein.

3.3. T1 transgenic tobacco plants showed increased drought tolerance

Seeds from transgenic tobacco lines 26 and 30 were placed in kanamycin medium until 2 leaves developed, then transferred to pots of yellow sand. To investigate the biological function of *GmEXP1*, these T1 generation plants were placed in an artificial drought condition for 3, 5, 7, and 9 days and were compared between experimental plots and the control plots, the transgenic plants and control nontransgenic plants (Figure 5).

To further investigate the role of *GmEXP1* in drought tolerance, we evaluated the root development of T_1 generation plants. When plants developed 4 or 5 true leaves, the root development was subjected to investigation after 3, 5, 7, and 9 days of artificial drought. The assessment criteria included the root length, volume, and dry mass (Figure 6).

Root morphology varied depending on the control or drought environment. In general, over 3, 5, 7, and 9 days of drought, roots all increased in length, dry mass, and

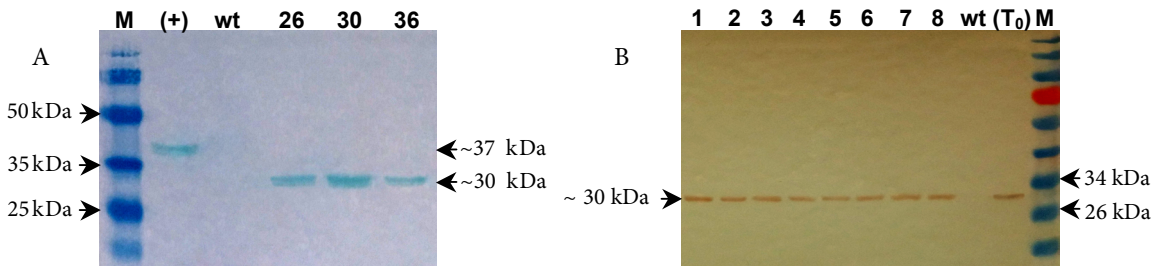

Figure 4. Western blot for recombinant expansin protein in transgenic tobacco plants T_0 (A) and T_1 (B). M: Standard protein ladder (20–120 kDa); 26, 30, 36: transgenic tobacco plants; wt: wild-type tobacco plant K326; (+): protein 37 kDa with c-myc tag; 1–4: T_1 transgenic tobacco plants from line $T_0$26; 5–8: T_1 transgenic tobacco plants from line $T_0$30; T_0: transgenic plants $T_0$26.

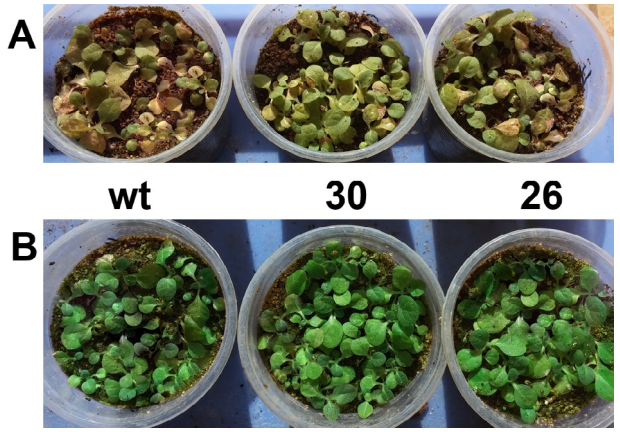

Figure 5. Wild-type and transgenic tobacco plants under drought (A) or normal watered condition for 5 days (B). 26, 30: transgenic tobacco plants from $T_0$26 and $T_0$30, respectively; wt: wild-type tobacco plants. Vertical bars represent standard error.

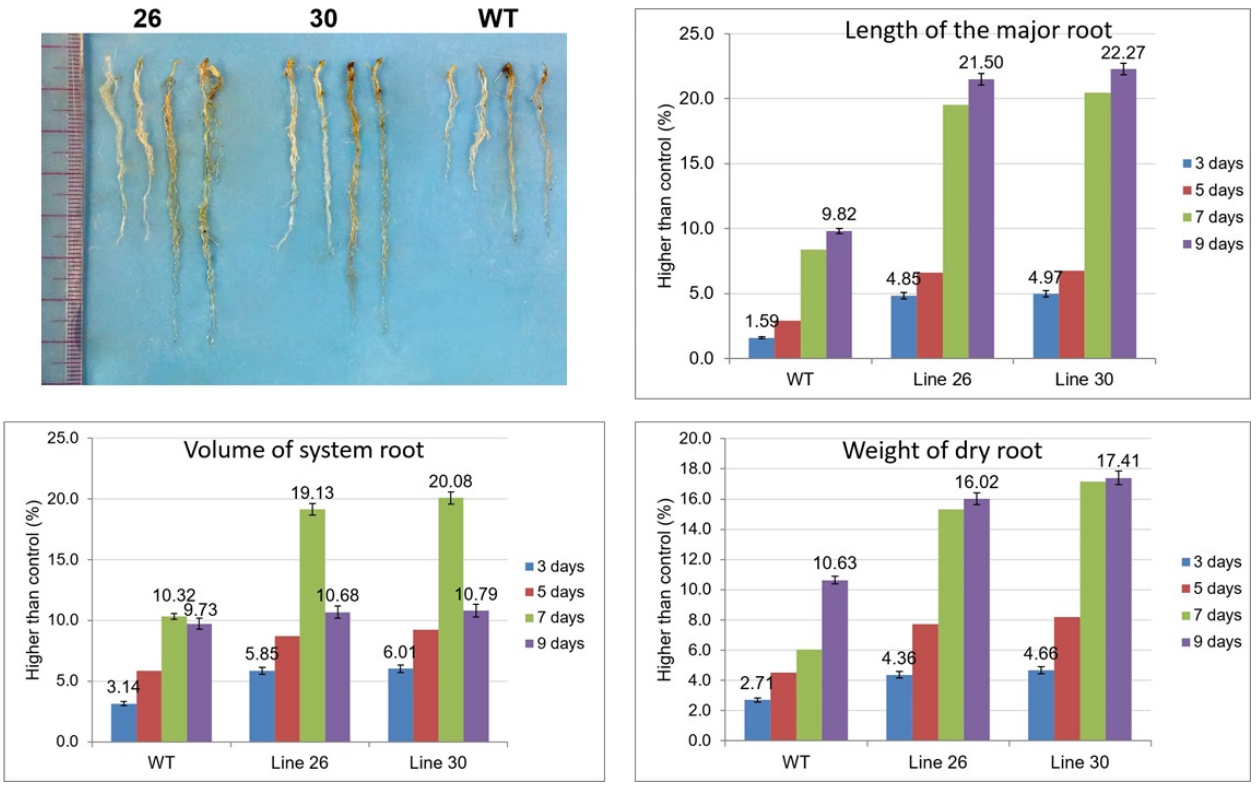

Figure 6. Root development (morphology, length, volume, and dry weight) in wild-type and transgenic tobacco plants compared to normal watered conditions. Vertical bars represent standard error.

volume compared to the roots of plants under the control watered condition. The length of the major roots from lines 26 and 30 increased from 4.85% to 22.27% compared to the control, while those of nontransgenic plants increased only from 1.59% to 9.82% after 9 days of experiment. The dry mass of the transgenic lines increased from 4.36% to 17.41% compared to the control, while in nontransgenic plants it increased only from 2.71% to 10.63% after 9 days of the experiment. The root volume from transgenic plant lines 26 and 30 remarkably increased from 19.13% to 20.08% compared to the control at day 7; nontransgenic plants increased up to 10.32%. The root volumes of all plants decreased at day 9, possibly due to the loss of water from the cells and the entire body caused by drought.

The above analysis has demonstrated the effect of the *GmEXP1* transgene on the size and volume of transgenic tobacco plant roots under 3, 5, 7, and 9 days of drought. It is clear that the *GmEXP1* gene from soybean genome was isolated, integrated in the tobacco genome, and inherited in the T_1 generation. The recombinant protein was shown to be stably expressed and have biological function in the ability to resist drought in transgenic plants.

4. Discussion

Climate change causes prolonged droughts and increased desertification in many parts of the world. This is one of the main causes of reduced productivity and quality of agricultural crops as well as shrinking farmable land. To combat these problems, it is urgently required to select and create agricultural crops that can resist and withstand adverse environmental conditions. Soybeans are relatively sensitive to external conditions and belong to the low drought tolerance group. Therefore, it is important to characterize the drought tolerance ability, as well as to study the physiological, biochemical, and molecular biological basis of drought tolerance in soybean plants, and to apply this improved ability. Traditional breeding methods have shown some results, but are very time-consuming, inefficient, and complicated and require large populations. To overcome these difficulties, advances in transgenic technology have helped to create transgenic soybean lines, which express important features such as improving drought tolerance, which has gained considerable achievements (De Ronde et al., 2004; Valente et al., 2009; Zhang et al., 2010; Li et al., 2013; De Paiva Rolla et al., 2014).

A study by Lee et al. (2003) showed that the *GmEXP1* gene, encoding an expansin protein, an important component in cell wall stretching, is abundant at the tip of the root and thus important for soybean root elongation. The mechanism of cell stretching by expansin proteins has been described. These proteins stick in the cell walls, breaking the link between the polysaccharides, which in turn loosens the cellulose fiber network, resulting in cell wall stretching under pressure (Cosgrove, 2000). Additionally, Guo et al. (2011) showed that the *GmEXPB2* gene was successfully isolated, which also encodes an expansin protein, and transferred into *Arabidopsis*. These transgenic plants are capable of increasing cell division and developing roots longer than in the control plants. Li et al. (2011) examined the drought resistance of transgenic tobaccos overexpressing *TaEXPB23*, which were cloned from wheat. The results indicated that the transgenic tobacco lines lost water more slowly than the wild-type plants under drought stress; their cells could sustain a more integrated structure under water stress than that of the wild type. In this study, *GmEXP1* isolated from the soybean cultivar SL1 was employed to design a construct expressing a recombinant expansin protein with a c-myc tag. We were able to detect the expression of the recombinant protein using the myc antibody in transgenic tobacco plants. The transgenic tobacco plants showed improved tolerance to experimental drought, indicating the potential to apply this approach to generate drought-tolerant soybean plants as well as other crops.

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