The Arabidopsis expansin gene (AtEXPA18) is capable to ameliorate drought stress tolerance in transgenic tobacco plants

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Abstract

Background Expansins are cell wall proteins loosening plant cell in pH-dependent manner. This study aimed to investigate the role of AtEXPA18 in different morphological, physiological, and cellular responses of transgenic tobacco plants to moderate and severe drought stress.

Methods and results Previously synthesized AtEXPA18 gene construct was successfully transferred to the tobacco plants through an agrobacterium-mediate transformation system. Upon obtaining the second generation, tobacco transgenic plants were confirmed by conventional polymerase chain reaction (PCR) technique alongside reverse transcription PCR (RT-PCR) using specific primers. Under drought stress, the transgenic lines showed remarkable growth and significantly improved based on morphological traits such as height and stem diameter, leaf area, leaf number, root dry weight, and Abscisic acid levels of leaves compared control plants. As a result, the Cytokinin content of transgenic plants has increased under severe stress levels. Notably, the area’s expansion for abaxial epidermal cells under the microscope confirmed in transgene cells compared with the -transgene cells.

Conclusion These results, altogether, could support the AtEXPA18 gene implication in cell expansion and improving tolerance capacity of transgenic crops under drought stress.

Keywords Agrobacterium tumefaciens · AtEXPA18 gene · Expansin · Drought stress · Tobacco

Introduction

Throughout the world, nearly all plant species, at least for a short period, are encountered with at least one biotic or abiotic stress. Among them, drought or water deficit stress is assumed to be one of the significant harmful factors, as it can negatively affect crop production of crops worldwide [1]. The plant cellular machinery routinely makes various morphological, physiological, biochemical, and genetic responses, as subsequent, to drought stress. In this way, most of these responses could independently/cooperatively help the plant body system minimize the scale of deleterious consequences of stress. In other words, some plant genotypes, even from single species, may exhibit high levels of tolerance domain so-called ”tolerant,” whereas others have restricted ability to resist so-called ”susceptible.” Hence, it is imperative to improve the tolerance domain of a given plant species under stress conditions, for instance, via introducing novel gene(s) to generate transgenic plants with improved tolerance domain and subsequently higher biomass [2].

Among candidate genes to manipulate for improving plant tolerance to abiotic stress, expansins recently have been attracted many researchers [3, 4]. Expansins are a class of cell wall proteins to promote the flexibility of the plant cell wall, mainly through loosening hydrogen bonds between the cellulose microfibrils and matrix polymer [5, 6]. These proteins, which firstly discovered in cucumber hypocotyls [7], usually encoded by a gene superfamily containing four sub-families: including α-expansin (EXPA), β-expansin (EXPB), expansin-like A (EXLA), and expansin-like B (EXLB) [3, 4, 8, 9]. It is currently well-adopted that they contribute to critical response mechanisms against various environmental cues, mainly through the regulation of cell growth and expansion [3, 4]. For instance, under water deficit stress, at
least two expansin genes were up-regulated at the maize’s root elongation zone and help root growth constantly continue at low water potential [10, 11]. In another investigation, Xu et al. [12] identified a heat-inducible expansin gene in Agrostis scabra, called AsEXP1, that could be a useful molecular marker for selecting heat-tolerant grass germplasm. Over-expression of the TaEXPB23 gene could enhance the drought resistance in tobacco plants [13]. Gao et al. demonstrated that the expression and activity of expansin proteins are induced under drought stress in wheat coleoptiles, suggesting the role of these proteins in response to water deficit circumstances [14].

Meanwhile, the regulative interaction between expansins and plant hormones has been suggested in cell enlargement, and cell wall changes under stress [15]. In a recent experiment, the association between Abscisic acid and expansin genes in bread wheat was investigated under abiotic stress. Furthermore, an increase of 20–60 fold in the amount of a β-expansin protein transcript (called Cim1) occurred by the addition of Cytokinin (Cyt) to Cyt-starved soybean suspension cultures [17]. Meanwhile, the exogenous application of Cyt could induce MeEXPA1 gene expression in Mello-tus Alba, demonstrating its potential regulatory roles on the expansins expression [18].

It is also believed that root hair distension and elongation thereof are often correlated with increased expansin activity, particularly EXPA8 and EXPA7 genes [19, 20]. Therefore, an AtEXP18 gene from Arabidopsis thaliana was initially cloned and subsequently transformed into tobacco plants (Nicotiana tabacum L.) to improve these plants’ tolerance under moderate or severe water deficit stress circumstances.

Materials and methods

Plant materials and transformation requirements

In this study, a high copy number pGEM-T Easy vector (Promega) and an expression vector of pBI121 (Clontech) carrying the kanamycin resistance gene was employed as plasmids of interest. The bacterium Escherichia coli strain DH5α was used to acquire competent cells and maintain the recombinant vector molecule. Tobacco plant leaves were used for inoculation by Agrobacterium tumefaciens strain LBA4404. The oligonucleotide primer sequences utilized for amplification of AtEXP18 gene were as follows: AtEXP18 FW 5′-GGATCCACGTTAAAAATGATCAAAATT-3′ (the BamHI site is underlined) and AtEXP18 RV 5′-GAG CTCTTAGTAAAATTAGCCTTGCTCTG-3′ (the SacI site is underlined).

Gene cloning and plant transformation

The gene construct of 35S::AtEXPA18, designed in our previous work [21], supplemented with a root-specific gene called AtEXPA18 were applied and subsequently cloned into the plant expression vector of pBI121 under the control of consensus CaMV 35S promoter and NOS terminator, which expressed throughout the plant (Fig S1). In the following, the concise construct was cloned into the A. tumefaciens strain LBA4404, the colonies with the recombinant plasmids were selected on the solidified LB medium (Lauria and Bertani) containing 25 µg/mL kanamycin and 50 µg/mL rifampicin. Then the PCR-validated colonies were used for tobacco leaf disk transformation [22].

The initial transgenic plants obtained from regeneration were then selected on Murashige and Skoog (MS) medium containing 50 µg/mL kanamycin (Kan) and 400 µg/mL cefotaxime (Sigma). After growing up in a monitored chamber at 20–22 °C, 40–60% relative humidity under a 16/8-h photoperiod at 100–120 µmol/m² s, the probable transgenic plants were transferred into the soil and grown in a greenhouse under 12/12-h light/dark with a full light intensity of 200–300 µmol/m² s at 25/22 °C (day/night). T1 generation seeds were separately obtained at the end of T0 generation plant growth and were sterilized and germinated on the selective MS medium with 50 µg/mL Kan to determine transgene inheritance and the number of inserted gene copies. The integrated gene’s segregation was determined for each line (i.e., five transgenic lines) by counting resistant and susceptible seedlings to Kan after 4 weeks of growth in Petri dishes. Further experiments were performed on the L2, L4, L5 lines with a single inherited transgene copy [23].

Drought stress treatments

In this step, 45-day-old transgenic and control tobacco seedlings were sown in pots containing 1.5 kg mixed soil (prepared with sand and mixed soil with leaf manure in ratio 1:2) in a greenhouse as described above. A 4 × 3 factorial experiment (i.e., four genotypes and three levels of water stress) in the form of a completely randomized design (CRD) with three replications was carried out in the greenhouse to impose drought stress on the transgenic plants. The two experiment factors consisted of plant materials (i.e., transgenic lines namely L2, L4, and L5 alongside one non-transgenic tobacco plant, as control), and drought stress treatments at three different levels [i.e., 40%, 60%, and 100% field capacity (FC) of pots soil]. Drought stress treatments were performed on 18-week-old tobacco seedlings with 15–18 leaves and continued 21-day long. The pots were
weighed daily and irrigated according to their corresponding FC.

**Conventional PCR and qRT-PCR**

Total genomic DNA was isolated from the green leaves of T₀ and T₁ transgenic tobacco plants using the CTAB method. The AtEXPA18 gene was amplified via PCR with the specific primers under the following amplification conditions: an initial cycle of 10 min at 94 °C, a 36-repeated cycle of 1.0 min at 94 °C, 1.0 min at 60 °C, and 2.0 min at 72 °C followed by a final extension step at 72 °C for 5 min. The resultant PCR products were separated on a 1.0% agarose gel and photographed by a gel documentation apparatus. Total RNA was extracted from the frozen tobacco leaves of T₁ generation with the Pbiozol™ reagent and treated with DNase I (RNase-free, Promega) to remove genomic DNA. Reverse transcription was performed using the primer oligo (dT)₁₅ and RevertAid™ M-MulV Reverse Transcriptase (Fermentas) at 42 °C for 60 min before.

**Measurement of morphological and physiological features**

Morphological characteristics of the tobacco plants, including stem height (cm), stem diameter (mm), leaf area index (LAI; cm²), and the number of leaves, were measured before and after drought stress treatment. In this case, the effects of the transgene expression on cell sizes were evaluated by excising the same age leaves from either transgenic or non-transgenic tobacco plants and dividing them into three distinct parts (i.e., basal, middle, and tip). The lower epidermis of leaves was taken and analyzed with an Axiophot microscope (ZEISS, Germany) after staining with Carmine. Subsequently, the photos were taken by a digital camera mounting to the microscope and connected to the computer and analyzed via Digimizer software (version 4.1.1.0). The areas of cells and cellular density (i.e., the number of cells per mm²) were calculated according to the following formula [23]:

\[
\text{Cell number} = \frac{\text{leaf area}}{\text{cell area mean}}
\]

\[
\text{Cell density} = \frac{\text{cell number}}{\text{leaf area}}
\]

Simultaneously, the same old leaf samples were separated and transferred into a −80 °C freezer for further molecular analyses. Lastly, at the end of the drought stress period, plant roots were elicited from the soil, washed carefully using distilled water, and oven-dried at 70 °C for 48 h to measure dry weight.

**Determination of plant pigments**

The measurement of total leaf chlorophyll content was conducted according to the method described by [24, 25]. In this context, 400 mg of fresh leaves of each sample were carefully fragmented into tiny segments in 80% acetone. The extracts were individually centrifuged at 8000×g for 5 min, then the total chlorophyll and carotenoids contents were determined in terms of the absorbance at the wavelengths of 470, 645, and 663 nm using Shimadzu UV-160 spectrophotometer. The calculation was carried out through the following formulae:

\[
\text{Total chlorophyll content} = \left[ 20.2 (\text{OD}_{645}) - 2.69 (\text{OD}_{663}) \right] \times \frac{V}{1000} \times W
\]

where V and W indicate volume of the extract and weight of leaf segments, respectively.

**Extraction, purification, and determination of plant hormones**

The extraction and purification of the phytohormones (i.e., ABA and Cyt) from the frozen young leaves were performed, as detailed below. Notably, measurement of both phytohormones was conducted only for L₄, L₅, and wild-types at only two levels of drought stress, including 100% FC and 40% FC.

Measurement of endogenous ABA levels was performed according to the protocol [26], with some minor modifications. In brief, ~1.5 g of the frozen tobacco leaves were finely ground using liquid nitrogen in a mortar and pestle, and 10 mL of 80% methanol was added together with 0.01 g of ascorbic acid and 0.01 g of polyvinyl pyrrolidone (PVP), actually to prevent oxidation reactions over-extraction. The homogenate was stirred overnight at 4 °C, centrifuged at 4000×g for 15 min, the supernatant was recovered and adjusted to pH 8.0, and centrifuged as before. The whole supernatant was adjusted to pH 2.5 and added 10 mL ethyl acetate. Then, the solution with the free ABA in ethyl acetate was collected, allowed to evaporate ethyl acetate, the residue sediment was dissolved in 1.0 mL solution containing 3% methanol and 0.1 M acetic acid, and filtered through a 45 µm membrane filter. 20 µL of the extract was injected and processed by HPLC (Unicam-crystal-200, England) equipped
with a reverse phase column (4.6 × 250 mm Diamonsic C18, 5 µm). It was eluted with a linear gradient of methanol-acetic acid (3–97%), at a flow rate of 4 mL/min. The detection was run at 260 nm with a diode array detector.

Measurement of endogenous Cyt levels was also conducted based on the method described by [27]. The HiQsil C18, 5.0 µm (4.6 × 250 mm) column was used to evaluate this phytohormone so that 20 µL of the extract was injected into the column and processed by HPLC apparatus. Washing solvent (deionized water: methanol (1:1) with 2.0% formic acid) was used at a 1 mL/min flow rate. Quantification was performed by comparing the peak areas with initial amounts of ABA and BA (99.97%, Sigma).

Statistical analysis

The data were statistically analyzed by IBM SPSS software (Multivariate Analyses of General Linear Model), and Duncan’s multiple range tests (p < 0.05) was used to determine critical values for comparisons between means. Also, the drawing of diagrams was performed by Excel software, 2013.

Results

Affirmation of Agrobacterium colonies with recombinant plasmids

All the transgenic agrobacterium-based colonies containing recombinant plasmids were successfully grown in the selective medium (Fig S2A). Moreover, the full-length AtEXPA18 gene (i.e., 1228 bp) was confirmed through colony PCR technique and extracted plasmids, indicating the presence of gene construct pBI::EXPA18 in the corresponding colonies (Fig S2B).

35S::AtEXPA18 tobacco lines

The insertion of AtEXPA18 into the tobacco genome was confirmed by PCR technique in five obtained plants at the first generation called T₀ tobacco lines (Fig S2C). The mature seeds of transgenic tobacco lines (called T₁) were successfully germinated at the selective MS medium (see above), actually owing to the 35S::AtEXPA18 construct expressing the kanamycin resistance gene, Neomycin phosphotransferase (nptII), whereas the wild type and non-transgenic tobacco plants were decayed after germination. The number of healthy: unhealthy tobacco seedlings illustrated that transgenic lines’ germination occurred nearly with 3:1 differentiation, demonstrating the insertion of one copy of the transgene into the plant genome (Fig S3), albeit transgenic L3 line showed 15:1 differentiation, exceptionally. After 28 days of growth in petri dishes, all the transgenic lines (T₁) and control plants were acclimated to soil conditions, and the results of RT-PCR confirmed the transgene transcription in transgenic tobacco lines, as the expected amplicon size of 789 bp was visualized (Fig S4).

Effect of water-deficit stress on morphological characteristics

The morphological measurements, including stem height and diameter of transgenic lines, were significantly higher than those of control plants (p < 0.01) under three levels of water deficit stress. Surprisingly, the transgenic line L2 exhibited the highest stem under three water deficit stress levels and the thickest stem only under control condition (Fig. 1A–C). After the application of drought stress, however, L2 and L4 had the thickest stem. Comparatively, the higher value of LAI was observed in all the three transgenic lines of L2, L4, and L5 under either normal or water shortage circumstances (p < 0.05; Fig. 2A), which could be due to high frequency of the leaves in transgenic lines compare to wild-type. Although, drought stress caused the significant decreasing of total LAI in both non-transgenic and transgenic plants, but this phenomenon was more remarkable in the former than the latter. Furthermore, all the three transgenic lines of L2, L4, and L5 produced higher number of leaves either under normal or water scarcity situations (p < 0.05; Fig. 2B). Similarly, it appears that the number of leaves less affected by drought stress in three transgenic lines rather than the wild-type tobacco plants (p < 0.05; Fig. 2B).

Effect of water-deficit stress on root dry weight

Because of AtEXPA18 gene activity in plant root, especially elongation [19], the experimental tobacco roots’ dry weight were measured. The results demonstrated a significant difference (p < 0.01) between transgenic and control tobacco plants. Indeed, all the three transgenic lines of L2, L4, and L5 produced higher root dry weight than the wild-type either under normal or water insufficiency circumstances (p < 0.05; Fig. 2C). It is worth mentioning that all the three transgenic lines of L2, L4, and L5 followed by wild-type plants overall could yield higher root biomass under severe drought and particularly moderate stress. This capacity could be due to the plant’s ability to adapt to the new situations (moderate drought stress), for instance, by employing elongated root systems to acquire more accessible soil moisture at the lower soil depths. However, root biomass not affected significantly in both
transgenic and non-transgenic tobacco plants under severe drought stress (Fig. 2C). Given the results, the root dry weights under moderate (60% FC) were higher than those calculated for the other stress levels (unlike stem height), demonstrating that the produced dry materials may have been more allocated into the roots than the stems under water deficit conditions. It should be also noted that transgenic plants had more root hairs than controls.

**Effect of water-deficit stress on total leaf chlorophyll content**

Considering ANOVA results, it seems that genotypes ($p \leq 0.05$) alongside different levels of drought stress ($p \leq 0.05$) could significantly vary in terms of the chlorophyll content (Table 1). As Fig. 2D shows, except for the L4 line, the other two L2 and L5 lines showed lower chlorophyll content than non-transgenic plants under water shortage conditions.

**Phytohormones**

**ABA and Cyt variations of leaf tissue of experimental tobacco plants under drought stress**

In this study, due to the close correlation between expansin genes and phytohormones, ABA and Cyt hormones were also isolated from upper extended two leaves of L4, L5, and wild-type tobacco plants under 100% FC and 40% FC, and analyzed then by HPLC apparatus. The results showed that ABA content in transgenic tobacco leaves was significantly ($p < 0.01$) higher than that of control plants at both non-stress and intensive drought stress levels (Table 2). Means comparison investigation revealed that the transgenic L4 line had the highest ABA content of leaf by 88.20 nmol/g FW at two drought levels (Fig. 3A). As Fig. 3A shows, an increment in ABA content from 100% FC to 40% FC in L2, L4, and control lines was 1.67, 1.42, and 2.14, respectively. Notably, albeit the ratio of ABA content in control lines was higher than L2 and L4, the absolute values of the phytohormone ABA in both
transgenic tobacco lines were remarkably higher than that of control, either under 100% FC or 40% FC. Unlike ABA, Cyt variation between transgenic and non-transgenic tobacco leaves wasn’t significant (Table S1), but there was a notable point in this phytohormone’s content at severe drought stress (40% FC; Fig. 3B). Under severe drought stress (40% FC), a little Cyt enhancement was observed in transgenic tobacco leaves, whereas the control plant leaves demonstrated a decrease of Cyt level compared with 100% FC (Fig. 3B).

**Table 1** ANOVA results for stem height, stem diameter, LAI, number of leaves, root dry weight, and total chlorophyll content

| SOV² | Dfᵇ | Stem height | Stem diameter | LAI | Number of leaves | Root dry weight | Total chlorophyll |
|------|-----|-------------|---------------|-----|------------------|-----------------|------------------|
| Genotypes (G) | 3 | 105.78** | 0.85** | 505.123* | 29.65** | 0.14** | 55.03* |
| Stress (S) | 2 | 30.88** | 0.19ns | 55,878.71** | 104.86** | 0.30** | 69.40* |
| G × S | 6 | 6.12ns | 0.08ns | 622.29ns | 0.94ns | .010ns | 25.94ns |
| Error | 24 | 11.83 | 0.08 | 1541.925 | 3.00 | 0.01 | 15.46 |

Both * and ** exhibit significant levels at \( p \leq 0.05 \) and \( p \leq 0.01 \), respectively, while “ns” means “not significant”

²Source of variation

ᵇDegree of freedom

**Table 2** ANOVA results for ABA, and Cyt contents

| SOV² | Dfᵇ | ABA | Cyt |
|------|-----|-----|-----|
| Genotypes (G) | 2 | 2882.47** | 59.46ns |
| Stress (S) | 1 | 2260.16** | 0.57ns |
| G × S | 2 | 164.00ns | 30.37ns |
| Error | 12 | 48.07 | 23.85ns |

Both * and ** exhibit significant levels at \( p \leq 0.05 \) and \( p \leq 0.01 \), respectively, while “ns” means “not significant”

²Source of variation

ᵇDegree of freedom
Effect of water-deficit stress on cellular levels characteristics

For investigating the effects of AtEXPA18 transgene expression on the cellular sizes, the areas of lower epidermal cells were calculated using a microscope (Table S2; Fig. 4). The results demonstrated that the average cell area and the leaf area of AtEXPA18-carrying plants were approximately 9% greater and 25% larger than those of control plants, respectively. The data also showed that the lower number of cells per unit area of the leaf surface is related to the enlargement of the cells in transgenic plants. In this case, transgenic plants had, on average, 6.02 cells per 1.0 mm² compared to the non-transgenic plants by 6.61 cells per 1.0 mm² (Table 1). Therefore, transgenic tobacco plants expressing the AtEXPA18 possessed extended walls and large partitions, possibly because of its function at cell walls compared with the non-transgenic plants.

Discussion

Expansins are involved in several biological functions, including plant growth, development, and environmental stress resistance [3, 4, 6, 28]. In this study, we investigated the effects of AtEXPA18 overexpression to improve drought tolerance by analyzing phenotype and hormone variation of T1 generation of transgenic tobacco lines under drought stress. In general, the overexpressing of the AtEXPA18 had positive effects on plant organ growth, which were in agreement with the earlier observations [19]. Also, under drought stress, transgenic plants had higher biomass than non-transgenic plants, agreeing with [13]. It has been reported that expansins activities occur mostly in young and undifferentiated cells, which normally lead to a growth in the stem length and diameter mainly via further expansion of these cells [29]. As it is known, cell division and cell expansion, as two vital events, play a significant role in the conversion of leaf primordium to mature leaf in plants, and after the cell division phase, the leaf mainly grows further through turgor-driven cell expansion, cell wall loosening and de novo synthesis of cell wall components [30]. Similarly, here, the overexpression of AtEXPA18 in cell expansion phase led to the generation of larger leaves in transgenic tobaccos as compared with the control plants. At the study of Pien et al. it was similarly indicated that the localized induction of expansin transgene expression on the flanks of tobacco vegetative meristems not only induced the appearance of leaf primordial, but also reiterated the entire process of leaf development and produced phenotypically normal leaves.
Interestingly, overexpressing the RhEXPA_4 in Arabidopsis caused some modification such as smaller, compact cells, fewer stomata, midvein vascular formation in leaf blade epidermal structure, which properly made them tolerant to abiotic stress [32]. Chlorophyll content, significantly was higher only in one transgenic line L4 compared to control under water deficit condition. In parallel with this, overexpression of EaEXPA1 in sugarcane caused higher content of chlorophyll under drought stress, indicating better performance of photosynthetic apparatus in transgenic lines [33]. Considering the vital function of expansin in cell wall loosen and division, the overexpression of EXPA19 may be caused cell size expansion, high content of chlorophyll and subsequently larger size of leaf in transgenic line compare to control [4]. Drought stress alters the pattern of assimilates allocation, as in most plants, it changes the biomass allocation pattern in favor of root, so the plant is able to use limited water supply effectively. Plants with the highest drought tolerant have more root weight, longer roots and more root/shoot ratio than susceptible cultivars. Considering that EXPA18 gene expression occurs in roots with high rate and causing root’s expansion [19], it was expected that transgenic tobaccos are able to grow more than control plants under stress condition, as the same results were deduced here.

Drought as environmental factor induces the production of ABA in roots which subsequently causes the stomatal closure and growth restriction. During alkaline trapping phenomena, which low ABA exerted from xylem and leaf apoplast to symplast, transporting more ABA into guard cells drastically promotes the orchestrating ability of stomatal aperture in response to abiotic stress. Accumulation of this hormone leads to ABA-dependent signaling pathway which vitally activates some important stress responsive genes by TFs such as Leucine zipper Domain (bZIP-type) in the form of dimer with ABA responsive elements. In addition, MYB, MYC factors that are in cis-element recognition site of Responsive to Dehydration 22 (RD22) overexpressed under drought stress and contributed in tolerance mechanism [34–36]. It was demonstrated that RhEXPA_4 promoter containing ABRE-related cis-elements made transgenic Arabidopsis plants high tolerant to drought and salt stress, indicating that EXPA might be implicated in ABA-dependent pathway [32]. Other signaling pathways including osmotic stress, calcium-dependent pathway, MAPK-mediated, proteolysis, and ROS involved in ABA-mediated responses to drought. NADPH oxidase genes including AtRbohD and AtRbohD were phosphorylated by SnRK2 activated by ABA in guard cells, indicating that ROS signaling and ABA have overlap which contribute in reaching these signals to antioxidant mechanism [34].

On the other hand, it is clear that ABA level is usually increased in plants during biotic or abiotic stress and plays a key role in plant signal transduction and response to these stresses [37]. Here, the results showed that ABA content in transgenic tobacco leaves was significantly higher than that of control plants at both non-stress and severe drought stress levels. Hence, due to changes in ABA content and differences between AtEXPA18 transgenic lines and control plants, it seems that ABA affects expansin genes only at the expression level. Although, under osmotic stress, ABA induced expansin activity it decreased the plasma membrane H+-ATPase activity and cell wall acidification which was unfavorable for expansin activity. Also, it should be mentioned that simultaneously increasing ABA (inhibits elongation) and expansin (loosening the cell wall) may be enhance the expansion, but could not compensate the other negative factor such as low cell turge induced by ABA [16]. Based on acid-growth theory, auxin increases the proton pump action which in turn raise up the H+ excretion into the apoplast [38]. In addition, it was demonstrated that auxin and ABA regulate the Arabidopsis H+ ATPase2 (AHA2), which pumping protons into extracellular space, through phosphorylation mediated by PP2C.D family of protein phosphatase. Small Auxin-Up RNA (SAUR), in contrast to ABA, heighten the phosphorylation of the AHA2 and then represses it that causes the elongation of cell [39]. Given the high content of ABA hormone in transgenic tobacco leaves, it could be hypothesized that the increasing amount of AtEXPA18 protein in the cell wall and the dual effects of this hormone on the protein activities disturbed the existing balance between them. As a result, the plant tends to increase its leaf ABA content, mainly in guard cells, in fact to keep the water under dry conditions.

Even though little information is available regarding the relationship between cytokinin hormone and expansin genes activities, several studies have been confirmed the potential relationships between each other. For instance, it has been reported that Cyt regulates a soybean β-expansin gene expression by a post-transcriptional mechanism [17]. In addition, numerous investigation has been recorded the role of exogenous application of Cyt and related expansin genes [15, 18, 40, 41]. Here Cyt content in the leaves of transgenic tobacco was increased after aggregation of AtEXPA18 protein at cell walls under severe drought stress, suggesting the potential role of expansin to improve this hormone content and subsequently plant tolerance versus drought stress circumstance.

**Conclusions**

In conclusion, our results indicated that intensified expression of an expansin gene could positively provoke the growth of different organs of transgenic tobaccos, and subsequently,
higher plat biomass could be acquired under water stress conditions. Meanwhile, analysis of plant hormones in transgenic lines confirmed the relationship between them and expansin genes activities, the exact evidence of this matter requires subsequent experiments. Finally, this gene’s overexpression could be recommended as a typical growth regulator, mainly to obtain transgenic plants with larger organs and extensive drought tolerance domain.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-021-06589-2.

Acknowledgement We kindly appreciated the University of Tehran for providing plant biotechnology lab and greenhouses.

Author contributions All authors equally made substantial contributions to the conception or design of the work, analysis, or interpretation of data. All authors approved this version to be published. All materials and data clearly reported and are accessible.

Funding Not applicable.

Declarations

Conflict of interest There is no conflict of interest.

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