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AGROBACTERIUM-MEDIATED TRANSFORMATION AND *IN VITRO* EVALUATION OF DEHYDRATION TOLERANCE IN TRANSGENIC TOBACCO HARBORING *Ca-AFP* GENE

Tran Thi Ngoc Ha✉**, Hoang Van Duong, Nguyen Huynh Cam Tu, Phan Tuong Loc**

Institute of Tropical Biology, Vietnam Academy of Science and Technology, 9/621 Hanoi Highway, Linh Trung Ward, Thu Duc City, Ho Chi Minh City, Vietnam

✉To whom correspondence should be addressed. E-mail: ttnha@itb.vast.vn

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SUMMARY

We have known that plant defensins have the main roles in biotic stress, especially antifungal activities. In addition, their functions on abiotic stress tolerance have been demonstrated on previous studies, but that information was still limited. *Ca-AFP*, a plant defensin gene derived from chickpea, was transformed into tobacco leaf disks by *Agrobacterium* method. After 16 weeks on selection media containing 15 mg/L hygromycin, some regeneration shoot lines were obtained. Four of them showed the positive bands on *hpt* gene and *Ca-AFP* gene by PCR screening. These lines were applied on *in vitro* evaluation of dehydration tolerance using rotary liquid culture, and the water-deficit stress was caused by 2% (w/v) PEG 6000. These four transgenic lines had better growth of adventitious roots (number of adventitious roots, total adventitious root length, and average of root length) and higher rate of biomass increase than the non-transgenic line. The result illustrated that the expression of *Ca-AFP* gene could mitigate the negative effect of water-deficit stress on transgenic tobacco lines under the *in vitro* stress condition. These transgenic tobacco lines are potential for water-deficit stress trials on greenhouse in the future.

Keywords: *dehydration tolerance, in vitro evaluation, PEG 6000, plant defensins, tobacco*

INTRODUCTION

Drought, which is one of the consequences of climate change, has negatively impacted human life, economic and social activities (Rojas, 2020). In particular, agriculture is very vulnerable, and food security is threatened in drought conditions. Because drought causes the reduction in arable land, yield, and quality of agricultural products (Rojas, 2020). By the time, drought has become more and more severe. It is estimated that 12 million hectares of land are lost because of drought and desertification annually (FAO, 2017). About 80% of losses in the agriculture sector of developing countries were caused by drought. In the case of Vietnam, more than 83% of arable land has been impacted by drought since 2015 (FAO, 2017). One of the solutions to keep sustainable agriculture in the 21st century is to develop the drought tolerant plant resources (FAO, 2017). For more than 20 years, genetically modified plants have been developed and have contributed to global sustainable agriculture, and food security (ISAAA, 2016, 2017; *National Academies of Sciences, Engineering, and Medicine*, 2016).

Ca-AFP gene from chickpea (*Cicer arietinum*) can encode an antifungal protein of 74 amino acids (Islam *et al.*, 2007). The results from aligning Ca-AFP protein's sequence to those of other crops' defensin proteins showed that Ca-

AFP is a plant defensin protein, contains conserved regions with eight common cysteine residues, which include four disulfide bridges (Islam *et al.*, 2007; Kumar *et al.*, 2019). The new function of Ca-AFP protein was demonstrated in 2019 that it could enhance the survival rate of transgenic *Arabidopsis thaliana* seedlings and the vitality of those transgenic plants under water-deficit stress (Kumar *et al.*, 2019). The overexpression of *Ca-AFP* in *A. thaliana* increases the accumulation of endogenous proline (Kumar *et al.*, 2019), which is an osmolyte as well as a ROS scavenger, and contributes to stabilize cell membrane structures (Hayat *et al.*, 2012; Liang *et al.*, 2013). However, the water-deficit stress tolerance of *Ca-AFP* gene is limited, experiments have been performed only in *A. thaliana* so far. It needs to expand the research on other species to understand more about *Ca-AFP* gene's properties.

Polyethylene glycol (PEG) is a type of hydrophilic polymer, can be used to create high osmotic pressures in solution. It is demonstrated that PEG having molecular weight over 4000 cannot enter the cell wall pores, and is not absorbed by root cells, so it is not toxic to plants (Muscolo *et al.*, 2014; Rubinstein, 1982). Therefore, PEG 6000 has been used to mimic the drought stress in plants because it causes the reduction of plant's water potential by osmotic stress (Ahmad *et al.*, 2020; Faisal *et al.*, 2019; Meher *et al.*, 2018; Yu *et al.*, 2017).

In this study, *Ca-AFP* gene was transferred into *in vitro* tobacco leaf disks via *A. tumefaciens*. Then, the *in vitro* water-deficit stress was performed on the transformants by using rotary liquid culture containing PEG 6000. This experiment aims to initially assess the dehydration tolerance of *in vitro* transgenic tobacco lines through some traits such as the growth of adventitious roots (number of adventitious roots, total adventitious root length, and average of root length), and the rate of biomass increase (rate of fresh mass increase, and rate of dry mass increase). These traits are often used to evaluate the *in vitro* effects of abiotic stress in plant species (Kumar *et al.*, 2019; Yu *et al.*, 2017).

MATERIALS AND METHODS

Plant materials and cultivation conditions

In vitro tobacco explants (*Nicotiana tabacum* cv. Petit Havana) were propagated in MS medium (Murashige, Skoog, 1962). All samples (leaf disks, shoots, and plants) were cultured at 25 ± 2°C with a photoperiod of 8-h light/16-h dark and light intensity of 3000 lux.

Assessment of hygromycin concentration using for screening transgenic shoots

In this study, we assessed the impact of hygromycin on the regeneration of shoot from *in vitro* leaf disks cultured in regeneration media (basal MS medium supplemented 1 mg/L BA) (Gallois, Marinho, 1995). Concentrations of hygromycin-B (Gibco®, Invitrogen, UK) in this assessment were in the range 0, 5, 10, 15, and 20 mg/L, and samples were sub-cultured every two-week.

Agrobacterium strain, T-DNA constructs and transformation

The complete coding sequence of *Ca-AFP* gene (DQ288897.2, GenBank at NCBI) was synthesized with two restriction enzyme (RE) sites *Bam*HI and *Sal*I at 5' and 3' ends (Genscript, Hong Kong). Then, *Bam*HI-*Ca-AFP*-*Sal*I fragment was cloned to the same RE sites of the pFF19 vector (Timmermans *et al.*, 1990). *Ca-AFP* gene is under the regulation of constitutive CaMV35S promoter and CaMV35S terminator of the pFF19 vector. After that, pFF19-Ca-AFP vector was digested with *Hind*III, and *Eco*RI and *Ca-AFP* cassette was excised and sub-cloned to *Hind*III/*Eco*RI sites of pCAMBIA1301 binary vector (Figure 1B) (Tran *et al.*, 2020). This expression vector – pCAMBIA1301-Ca-AFP – was introduced into *Agrobacterium tumefaciens* EHA105 by freeze-thaw method (Weigel, Glazebrook, 2006).

A. tumefaciens harboring pCAMBIA1301-Ca-AFP vector (Figure 1B) was used for transformation in tobacco. In addition, *A. tumefaciens* harboring backbone pCAMBIA1301 binary vector (Figure 1A) was

also used for transformation experiment to create the empty transgenic control. *A. tumefaciens* strain was cultured in liquid LB medium supplemented with kanamycin and rifampicin by incubating at 28°C and shaking at 200 rpm overnight. Before the infection, cultured *A. tumefaciens* solution was centrifuged at 4000 rpm for 10 minutes. Then, the pellet was re-suspended in liquid ½ MS medium supplemented with 100 µM acetosyringone, and the optical density (OD_{600}) was in the range of 0.8–1.0. The leaf disks (1 cm x 1 cm) from 1-month-old *in vitro* tobacco explants were soaked in *Agrobacterium* solution for 15 minutes. After that, the excessive bacteria in infected samples were absorbed by aseptic filter papers. Then, infected samples were co-cultured in regeneration media (basal MS medium contains 1 mg/L BA) supplemented with 100 µM acetosyringone in the dark at 28°C for 3 days (Gallois, Marinho, 1995).

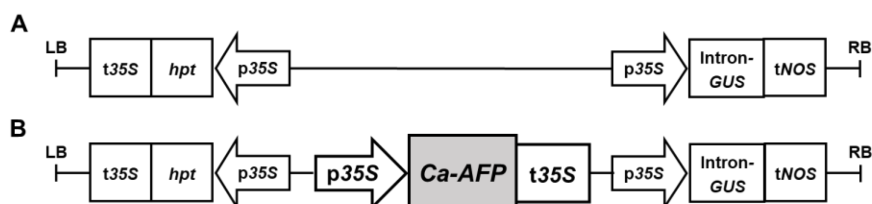

Figure 1. T-DNA constructs. (A) T-DNA of pCAMBIA1301; (B) T-DNA contains *Ca-AFP* gene. *hpt*, *hygromycin phosphotransferase* gene; *Ca-AFP*, *anti-fungal protein* gene derived from *C. arietinum*; *Gus*, *β-glucuronidase* gene; p35S, CaMV35S promoter; t35S, CaMV35S terminator; tNOS, nopaline synthase terminator. LB and RB, left and right border.

Selection of transformed samples

After the co-culture period, infected leaf disks were gently shaken in liquid ½ MS medium containing cefotaxime to eliminate *Agrobacterium*. Then, they were selected in regeneration media supplemented 200 mg/L cefotaxime and hygromycin, and were transferred to fresh media every two-week, until the regenerating shoots were over 2 cm in height. After that, they were moved to MS media containing 200 mg/L cefotaxime and hygromycin to develop and rooting.

PCR analysis

Firstly, to confirm the absence of *A. tumefaciens* on hygromycin-resistant shoots, half of the leaf was cut and incubated in 1.5 mL tubes (containing 1 mL LB) at 37°C and shaken 200 rpm for one hour. After that, leaves were discarded and 1 mL solution was centrifuged at 3500 rpm for 10 minutes to get pellets. Then, pellets were resuspended in 100 µL LB and spread on LB plates. These plates were incubated at 28°C for 48 hours. No colony appearing on these plates indicated that *A. tumefaciens* did not remain on resistant shoots.

Leaves of hygromycin-resistant shoots were collected and genomic DNA was extracted by using GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Scientific, USA). PCR amplification was carried out with Swift™ Maxi Thermal Cycler (ESCO, Singapore) according to the following step-cycle program: (1) pre-incubation at 95°C for 3 minutes, followed by 35 cycles of denaturing at 95°C for 60 seconds, and annealing at 54°C for 60 seconds and extension at 72°C for 2 minutes each cycle for *hpt* gene; and (2) pre-incubation at 95°C for 3 minutes, followed by 35 cycles of denaturing at 95°C for 90 seconds, and annealing at 65°C for 90 seconds and extension at 72°C for 2 minutes each cycle for *Ca-AFP* gene. The primer pairs for PCR were the following; 5′-CGTCGTTCGAGAAGT TTC -3′ and 5′-TACTTCTACACAGCCATC -3′ for *hpt* gene and 5′-CGCGGATCCATGGCGAGGTGTGAGAATT TGGCT -3′ and 5′-TGCTCTAGAATGGCGAGGTGTGAGAATT

TGGCT -3′ for *Ca-AFP* gene (El-Siddig *et al.*, 2009; Zhang *et al.*, 2015).

Evaluation of PEG 6000 concentration using for assessment of dehydration tolerance of *in vitro* transgenic tobacco

Node carrying one axillary bud was cut from two-month-old *in vitro* tobacco plants and transfer to fresh MS medium (Figure 2). When axillary buds were about 2 cm in height, they were cut and used as material for the evaluation experiment (Figure 2). Each non-transgenic shoot was put in one 250 mL flask containing 20 mL liquid MS medium (contains 30 g/L sucrose), shaking at 80 rpm (Huynh *et al.*, 2014). In this study, the dehydration tolerance experiment has three periods, namely Acclimation (the first two-day) without PEG, Osmotic stress/ Dehydration stress (the next 21-day) with PEG 6000 (0, 1, 2, 3, and 4% w/v), and Recovery (the last two-day) without PEG. The fresh mass of each shoot was weighed at the beginning of the acclimation period (starting point) and at the end of recovery period (endpoint). The rate of fresh mass increase (FM) was calculated as the formula:

$$\mathrm{FM} = \frac{\text{Fresh mass at endpoint}}{\text{Fresh mass at starting point}}$$

The number of shoots forming roots; and the number of adventitious roots and total length of adventitious roots of each sample were measured at the end of the recovery period (endpoint).

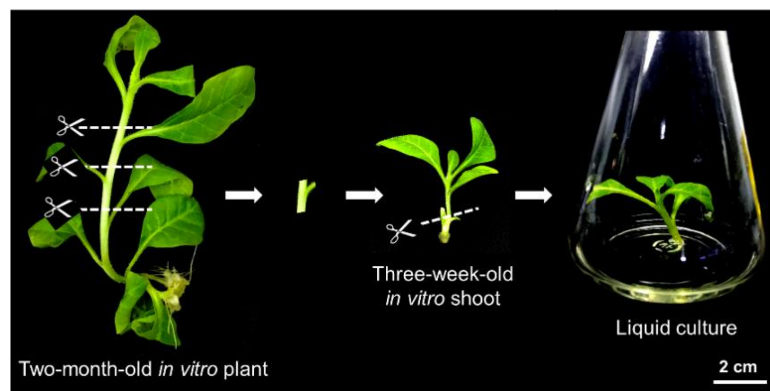

Figure 2. Preparing *in vitro* tobacco shoots for dehydration experiment.

Evaluation of dehydration tolerance of *in vitro* transgenic tobacco lines

This experiment was conducted in the same way as the above dehydration tolerance experiment (acclimation - osmotic stress - recovery). Liquid MS medium containing PEG 6000 in 2% (w/v) of concentration was used for the osmotic stress period. Explant materials were 2-cm shoots from tobacco transgenic lines/empty vector line and non-transgenic line. In addition, the control experiment was carried out with liquid MS medium (without PEG). The fresh liquid MS medium was replaced at the time corresponding to the start of the osmotic stress and recovery periods.

At the starting point (the start of the acclimation period), the fresh mass of each shoot was weighed. However, to get the dry mass of each shoot at starting point, we used the below formulas. With eighteen 2-cm shoots, each shoot was weighed the fresh mass. Then, they were incubated at 50°C for 48 hours to get the dry mass of each shoot. The ratio of dry mass and fresh mass (DF) was calculated for each shoot and got the average value. The average of DF was performed for each line (transgenic lines, empty vector line, and non-transgenic line).

$$\text{Ratio of dry mass and fresh mass (DF)} = \frac{\text{Dry mass of each shoot}}{\text{Fresh mass each shoot}}$$

$$\text{Dry mass at starting point} = \text{Fresh mass at starting point x Average of DF}$$

At the endpoint (the end of recovery period), fresh mass and dry mass of each sample were weighed. The number of adventitious roots and total length of adventitious roots of each sample were also measured. The rate of dry mass increase (DM) and the average of root length (ARL) were calculated as the below formulas:

$$\text{DM} = \frac{\text{Dry mass at endpoint}}{\text{Dry mass at starting point}} \qquad \text{ARL} = \frac{\text{Total adventitious root length at endpoint}}{\text{Number of adventitious roots at endpoint}}$$

Statistical analysis

The data were subjected to a statistical analysis using either one-way analysis of variance (ANOVA) or the split plot analysis of variance that was performed by using R ver. 3.6.0 software (2019-04-26) and/or Microsoft Excel 2016. The Tukey-Kramer multiple comparisons test was used as necessary, with R.

RESULTS AND DISCUSSION

Effect of hygromycin on *in vitro* tobacco leaf disks

In this experiment, after four weeks, most of leaf disks on the treatment of 15 and 20 mg/L hygromycin turned yellow-brown in color, and no shoot was induced in the edges of leaf disks. After that two weeks, all samples on the treatment of 20 mg/L hygromycin died without induced callus (Figure 3E, F). Therefore, this assessment was stopped and data of all treatments were recorded after six weeks of testing. The results illustrated that shoot regeneration rates on the control (without hygromycin) and 5 mg/L hygromycin treatment were about 99% and 90% respectively (Figure 3A). However, the height of regeneration shoots on these treatments was clearly different. In the control, shoots were about 10 mm in height and they developed on the entire leaf disk surface (Figure 3B). On the other hand, in 5 mg/L hygromycin, shoots only regenerated at the edges of the leaf disks and their height was lower than 4 mm (Figure 3C). Regeneration rates decreased sharply to 31.7% and 0.6% on treatment of 10 mg/L and 15 mg/L hygromycin in that order (Figure 3A, D, E). Soft calli were induced at the edges of the leaf disks on 15 mg/L hygromycin treatment, but shoots could not regenerate (Figure 3E). Results showed that 15 m/L hygromycin can inhibit the shoot regeneration of more than 99% of leaf disks (Figure 3A). Consequently, 15 mg/L was chosen as the concentration of hygromycin to screen transgenic shoots regenerating from *in vitro* tobacco leaf disks. This concentration is lower than previous reports, they used hygromycin in the concentration of 25-30 mg/L to screening transformants regenerating from *in vitro* tobacco seedlings' leaves (Pathi *et al.*, 2013; Vivek *et al.*, 2013). Those differences may be caused by different kinds of materials. Seedlings leaves (young tissue) are usually stronger and have a higher shoot regeneration capacity than *in vitro* mature leaves (old tissue) (Bhojwani, Dantu, 2013; Ikeuchi *et al.*, 2016).

Selection of transformed tobacco shoots and PCR detection

After 3 days of the co-culture period, infected leaf disks were sub-cultured in regeneration media supplemented with 15 mg/L hygromycin every two-week until forming resistant shoots. Small shoots regenerated in the edges of leaves after four weeks on selection media (Figure 4A). The height of some regeneration shoots reached 6 mm at the 7th week (Figure 4B). After ten weeks of selection on regeneration media, 2-cm-shoots were moved to propagation media (MS basal medium) containing 15 mg/L hygromycin for rooting and

developing to *in vitro* plants. After six weeks, in order to make sure that these regeneration shoot lines were the transformants, nodes carrying one lateral bud were cut from these explants and put in propagation media supplemented 20 mg/L hygromycin for six weeks. The results were obtained that nodes from hygromycin-resistant shoots could grow up and rooting (Figure 4C), but nodes from non-transgenic line turned brown and died after six weeks on MS media containing 20 mg/L hygromycin (Figure 4D).

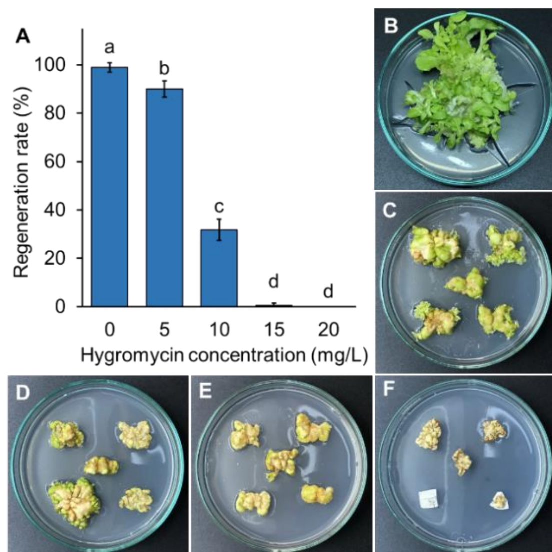

Figure 3. Effect of hygromycin on shoot regeneration from *in vitro* tobacco leaf disks. (A) Shoot regeneration rate of *in vitro* tobacco leaf disks on media containing different concentrations of hygromycin; (B–F) Shoot regenerating from tobacco leaf disks on different concentrations of hygromycin; (B) Without hygromycin (control); (C) 5 mg/L hygromycin; (D) 10 mg/L hygromycin; (E) 15 mg/L hygromycin; (F) 20 mg/L hygromycin. All data were recorded after 6 weeks in shoot regeneration media. Error bar in (A), Standard error (taking 30 samples in each treatment with three replications). The different letters on the top of the bars indicate the significant differences among treatments by the Tukey-HSD test (α = 0.05).

After the selection, nine shoot lines, which regenerated from nine independent samples, were obtained from 129 infected leaf disks. DNA genome was extracted from leaves of these nine lines. PCR analysis was carried out by using specific primer pair of selectable maker gene (*hpt* gene). The results indicated all of nine lines were transgenic lines, which had the positive band of 800 bp fragments of *hpt* gene. However, there are only four lines that had the normal growth and phenotype compared to the non-transgenic line when they were cultured on MS media without hygromycin.

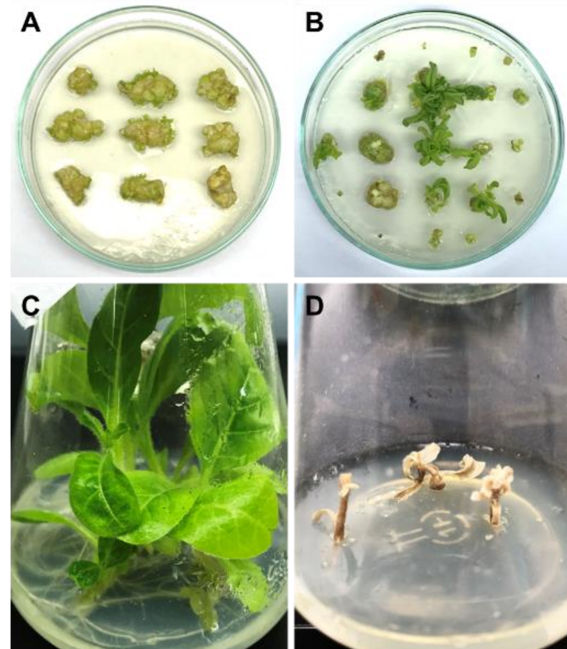

Figure 4. Results of the selection of infected tobacco leaf disks on media containing hygromycin. (A, B) Selecting infected leaf disks on shoot regeneration media containing 15 mg/L hygromycin 4 weeks (A) and 7 weeks (B) after infection; (C, D) Selecting regeneration shoots on propagation media containing 20 mg/L hygromycin for 6 weeks: infected samples (C), non-transgenic samples (D).

The insertion of *hpt* gene and *Ca-AFP* gene into DNA genome of four transgenic lines were demonstrated by PCR analysis. The 800 bp band of *hpt* gene and 250 bp band of *Ca-AFP* gene were observed in agarose gel after electrophoresis (Figure 5). Four transgenic tobacco lines, namely 1-3, 2-3, 3-2, and 4-2, would be plant materials for the assessment in dehydration stress by a liquid solution of PEG 6000.

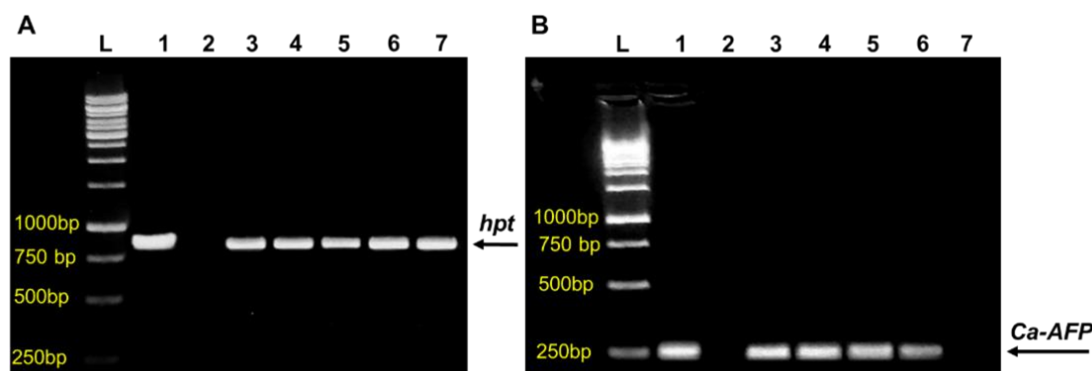

Figure 5. PCR analysis of genomic DNA from the transformed and non-transformed tobacco explants. (A) Amplification of the selectable marker gene (*hpt*) (800 bp); (B) Amplification of the target gene (*Ca-AFP*) (250 bp). Lane L: GeneRuler 1kb DNA Ladder, ready-to-use (Thermo Scientific, USA); 1: Positive control (plasmid DNA); 2: Non-transgenic tobacco line; 3–6: Independent transgenic tobacco lines harbor *hpt* gene, and *Ca-AFP* gene (1-3, 2-3, 3-2 and 4-2 lines); 7: Transgenic tobacco line carries empty vector.

Effect of PEG 6000 on the *in vitro* tobacco shoots

Acclimation time was the period for explants to adapt to the rotary liquid culture system. Coming to the osmotic stress period, explants formed roots after 5 days, 10 days, and 12 days on the control treatment, 1%, and 2% (w/v) PEG 6000 respectively. No root was observed on the treatment of 3% and 4% PEG 6000 at the end of osmotic stress period. After recovery period finishing, roots were recorded in some explants of 3% PEG treatment, but no root in the 4% PEG treatment.

Table 1. The growth of non-transgenic tobacco explants on media containing different concentrations of PEG 6000.

Concentration of PEG 6000 (% w/v)	Rooting rate (%)	Rate of fresh mass increase	No. of AR	Total AR length (mm)
0	100	8.0 ± 0.3^{a}	10.8 ± 0.4^{a}	763.7 ± 15.0^{a}
1	100	5.0 ± 0.2^{b}	9.1 ± 0.4^{a}	147.8 ± 18.9^{b}
2	100	3.9 ± 0.4^{bc}	3.8 ± 0.3^{b}	46.4 ± 7.8^{c}
3	48.1 ± 3.7	3.7 ± 0.2^{c}	0.9 ± 0.5^{c}	8.7 ± 4.5^{c}
4	0	3.4 ± 0.1^{c}	0	0

AR: adventitious root. Values were expressed as the mean ± SE (standard error), taking 9 samples in each treatment with three replications. The different letters on each column showed the significant differences on values among treatments by the Tukey-HSD test (α = 0.05).

At the end of this experiment (endpoint), all samples were alive and grew up (Figure 6). Rooting rate on treatments of 0-2% PEG and 3% PEG was 100% and around 50% in that order. Besides, 4% PEG inhibited rooting of all samples (Table 1). The results indicated that the increase of PEG concentration inhibited the growth of *in vitro* shoots, caused the decline in the "Rate of fresh mass increase". In particular, the ability to form and develop roots was negatively influenced by the increase in osmotic pressure of the culture solution, which caused the decrease in the water potential of root cells (Table 1 and Figure 6). On the solution of 2% PEG 6000,

although 100% explants could form roots, "Number of adventitious roots" went down by 64% to 3.8 compared to 10.8 in the control treatment (Table 1). Especially, "Total adventitious root length" plunged by 93% to 46.4 mm as opposed to those in the control treatment (Table 1). The deterioration of the adventitious root system caused a significant reduction in nutrient and water uptake and decreased the plant's vigor. Therefore, the "Rate of fresh mass increase" was significantly declined to only 3.9, which dropped to under 50% compared to those in the control treatment (Table 1), and some parts of leaves turned yellow (Figure 6).

According to the above results, 2% (w/v) PEG 6000 is the suitable concentration for assessment in dehydration tolerance of *in vitro* transgenic tobacco lines in next experiment. This result is similar to other reports that PEG 6000 was used to mimic drought stress in concentration of 1.2% (w/v) for *Populus tremula* x *Populus tremuloides* (Yu *et al.*, 2017), and 1.5–4.5% (w/v) for *A. thaliana* seedlings (Kumar *et al.*, 2019).

Figure 6. Phenotype of *in vitro* non-transgenic tobacco explants on different concentration of PEG 6000

Assessment on *in vitro* dehydration tolerance in transgenic tobacco lines

Four transgenic lines (TG) carrying *Ca-AFP* gene (namely, 1-3, 2-3, 3-2, and 4-2), one transgenic line harboring empty vector (EV), and one non-transgenic line (NT) were used as plant resources for the *in vitro* evaluation in dehydration tolerance caused by 2% (w/v) PEG 6000. In addition, the control treatment, non-stress condition, was parallel carried out with these transgenic/non-transgenic lines.

In stress condition, all samples formed adventitious roots after 10-12 days in osmotic period. The expression of *Ca-AFP* gene was indirectly showed through the development of adventitious root of TG lines. At the endpoint, "Number of adventitious roots" and "Total adventitious root length" of TG lines were remarkably higher than those of EV line and NT line. "Number of adventitious roots" of 1-3 line and 4-2 line were 2.7 and 3.9 times higher than those of EV and NT lines respectively (Figure 7A). Particularly, 2-3 line and 3-2 line had more 4.4 times adventitious roots number than EV and NT lines (Figure 7A). Similarly, "Total adventitious root length" of 1-3 line and 4-2 were 3.0 and 2.3 times longer than those of EV and NT lines in that order (Figure 7B). Those values of 2-3 line and 3-2 line also significantly greater 5.8 times than those of the control (Figure 7B). As it shown, 2-3 line and 3-2 line had the best growth of adventitious root system among transgenic lines (Figure 7A, B). In addition, "Average of root length" of TG lines were also longer than those of NT and EV lines, but there was no significant difference among them (Figure 7C).

Moreover, the recorded data shown that "Rate of fresh mass increase" and "Rate of dry mass increase" of TG lines were higher than

those of EV line and NT line, but it was not a significant difference, except "Rate of fresh mass increase" of 4-2 line and "Rate of dry mass increase" of 3-2 line (Figure 7D, E; Figure 8A). The better development of the adventitious root system of TG lines contributed to keeping the balance of water uptake from roots and water evaporation from leaves. Therefore, AFP-transgenic lines could mitigate the negative effects of water-deficit stress to absorb more water and nutrients, so they could get better growth of biomass than the controls.

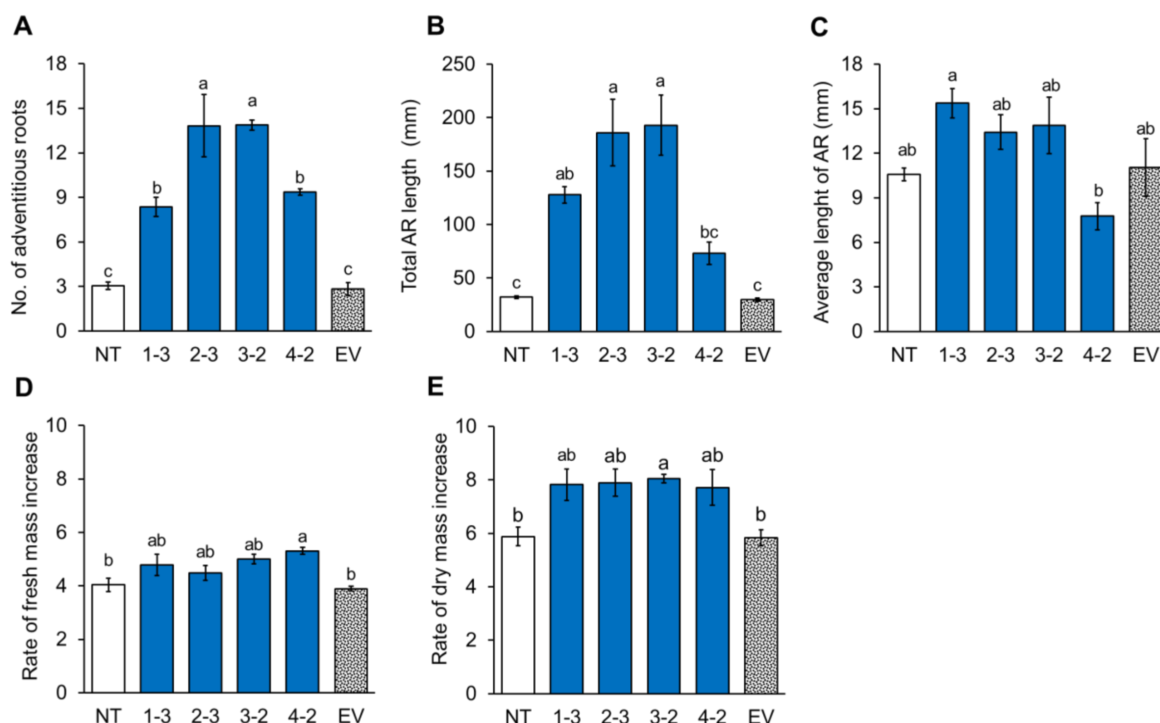

Figure 7. The growth of *in vitro* tobacco lines under dehydration stress condition. Stress condition was caused by 2% PEG 6000. AR: adventitious root; NT: Non-transgenic tobacco line; 1-3, 2-3, 3-2, and 4-2: Independent transgenic tobacco lines harbor *Ca-AFP* gene; EV: Transgenic tobacco line carries empty vector. Error bar: Standard error (taking 9 samples in each treatment with three replications). The different letters on the top of the bars indicate the significant differences on among lines by the Tukey-HSD test (α = 0.05).

On the other hand, in non-stress condition, there was no abnormal phenotype observed on four TG lines (Figure 8B and Figure S1). One-way ANOVA of data from "Number of adventitious roots", "Total adventitious root length", "Average of root length", "Rate of fresh mass increase", and "Rate of dry mass increase" illustrated that there was no remarkable difference among assessed lines (TG, EV, and NT lines) (Table S1). It is illustrated that the expression of *Ca-AFP* gene does not cause any negative effect on morphology of transgenic lines in non-stress condition, which is similar to the report of transgenic *A. thaliana* harboring *Ca-AFP* gene (Kumar *et al.*, 2019). This important feature is often evaluated in transgenic plants because some abiotic stress-related genes can increase the tolerance of transformants in stress condition, but they cause abnormal growth in non-stress condition. For instance, a reduction of leaf size as a dwarf phenotype has been observed in many transgenic plants when overexpressing *DREB/CBF* genes (Achard *et al.*, 2008; Huang *et al.*, 2009; Li *et al.*, 2012; Suo *et al.*, 2012).

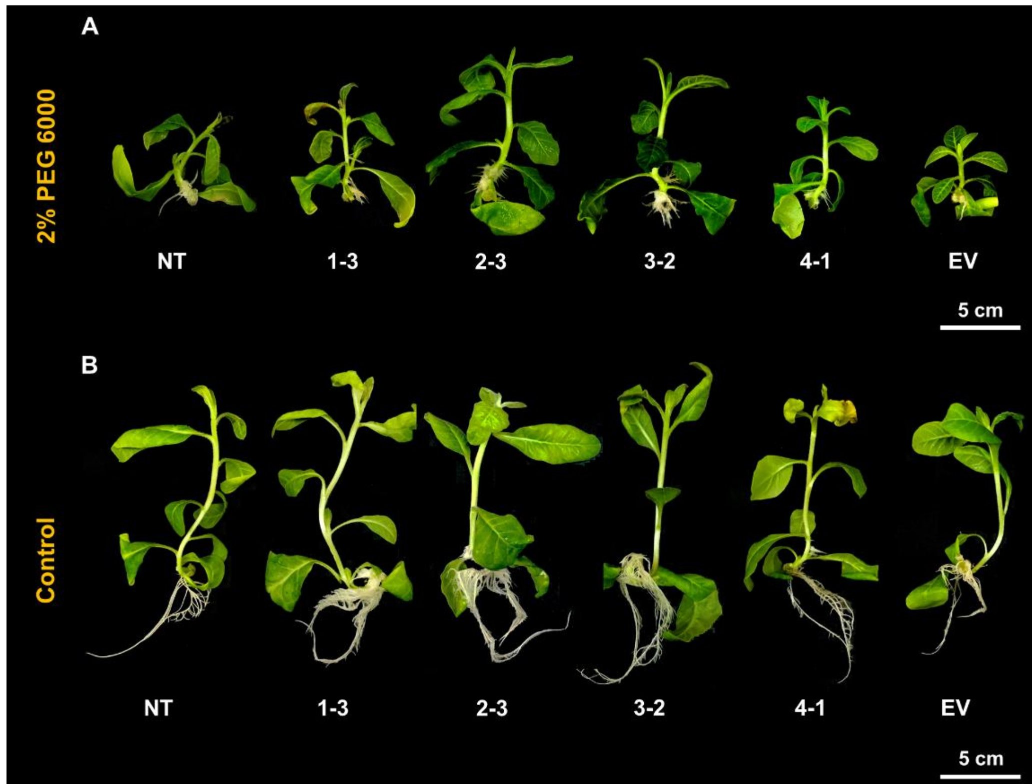

Figure 8. The phenotype of *in vitro* tobacco lines under dehydration stress condition and non-stress condition. (A) Dehydration stress condition was caused by 2% PEG 6000; (B) Non-stress condition. NT: Non-transgenic tobacco line; 1-3, 2-3, 3-2, and 4-2: Independent transgenic tobacco lines harbor *Ca-AFP* gene; EV: Transgenic tobacco line carries empty vector.

The above results revealed that the expression of *Ca-AFP* gene can increase the *in vitro* dehydration tolerance of four transgenic tobacco lines compared to the non-transgenic line. The adventitious roots system of transgenic lines is stronger and can expand longer in osmotic stress condition. However, *Ca-AFP* gene does not cause a negative impact on the morphology of transgenic lines.

CONCLUSION

Four transgenic tobacco lines harboring *Ca-AFP* gene, namely 1-3, 2-3, 3-2, and 4-2, were obtained by screening infected leaf disks on selection media supplemented 15 mg/L hygromycin. These transgenic lines were assessed the *in vitro* dehydration tolerance on rotary liquid culture by using MS media containing 2% (w/v) PEG 6000. The results indicated that four transgenic tobacco lines have higher dehydration-tolerance level than the non-transgenic line. They showed the better growth of shoots (rate of biomass increase) and wider/ longer adventitious root system. These four transgenic tobacco lines are potential for the greenhouse trial to evaluate more drought stress-related traits in the future.

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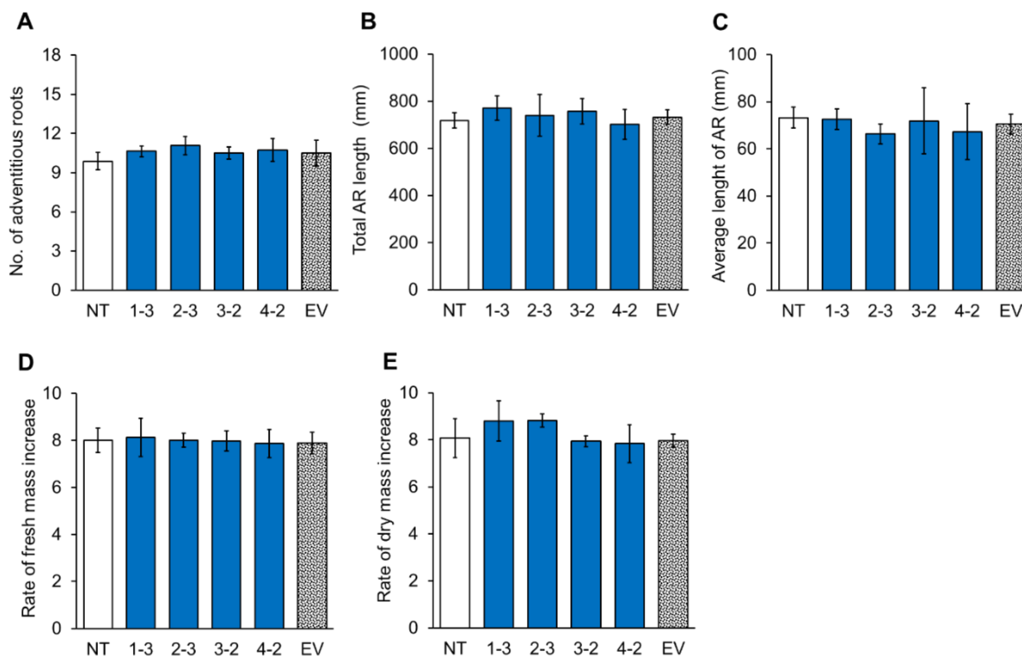
SUPPLEMENTARY



Suppl. Figure S1. The growth of *in vitro* tobacco lines under non-stress condition. AR: adventitious root; NT: Non-transgenic tobacco line; 1-3, 2-3, 3-2, and 4-2: Independent transgenic tobacco lines harbor *Ca-AFP* gene; EV: Transgenic tobacco line carries empty vector. Error bar: Standard error (taking 9 samples in each treatment with three replications).

Suppl. Table S1. Analysis of variance of dehydration stress-related traits of *in vitro* tobacco lines in non-stress condition.

Testing objects	Source	Df [a]	Sum Sq [b]	F value [c]	Pr(>F) [d]	
Number of adventitious roots	Line [e]	5	2.338	0.305	0.901	ns [f]
	Residuals	12	18.407			
Total adventitious root length	Line [e]	5	9694	0.201	0.956	ns [f]
	Residuals	12	115524			
Average length of adventitious root	Line [e]	5	114.2	0.159	0.973	ns [f]
	Residuals	12	1722.4			
Rate of fresh mass increase	Line [e]	5	0.103	0.023	0.999	ns [f]
	Residuals	12	10.633			
Rate of dry mass increase	Line [e]	5	3.022	0.527	0.752	ns [f]
	Line [e]	12	13.771			

[a] Degrees of freedom. [b] Sum of squares. [c] Variance ratio against error. [d] Probability of F-value. [e] Tested difference among the six lines. [f] Not significant (α=0.05)