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THE ACTIVITY OF *ARABIDOSPIS* DLL PROMOTER IN TRANSGENIC TOBACCO PLANTS UNDER WATER STRESS CONDITIONS

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ABSTRACT

In this work we used the Cre/*lox*P recombination system to study the activity of the *Arabidopsis DLL* promoter under water stress treatment. For this, the T-DNA containing the Cre/*lox*P self-excision recombination cassette was introduced into tobacco genome via *A. tumefaciens* LBA 4404. The expression of the *cre* gene was regulated by the *DLL* promoter. On activity of the *DLL* the Cre recombinase was expected to remove Cre/*lox*P cassette. Transgenic nature of regenerated transgenic T_0 tobacco plantlets was proved by GUS and PCR analyses. The selected 10 transgenic T_0 plants were subjected to the water stress analyses under *in vitro* as well as under *in vivo* conditions. The osmotic stress experiments were performed with 10 % PEG and 100 mmol.l^{-1} mannitol (individually). The activity of the *DLL* was evaluated after 24 hours. For drought stress experiments, the watering was withheld for 10 days. The activity of the *DLL* was monitored using PCR approach. Under given abiotic stress conditions, no activity of the *DLL* was observed. The *DLL* promoter remained stable. It points out the *DLL* as the promoter with precise control of the gene expression with wide usability in plant biotechnology.

Keywords: Abiotic stress, *Agrobacterium tumefaciens*, *Arabidopsis thaliana*, β-glucuronidase gene, Cre recombinase, *DLL* promoter

INTRODUCTION

Plant biotechnology offers new possibilities to prepare crops with improved properties. However, often commercial application is hampered by the lack of utility promoters that can drive gene expression in a tissue specific or temporary controlled manner. Besides, many tissue-specific promoters are also inducible by different abiotic/biotic stresses. The potential ectopic activity of the plant promoters should be carefully considered. Occasionally, premature transgene expression can have harmful effect on the host cell. For example, in approaches focused on the male-sterile transgenic plants using the barnase gene **(Roque *et al.*, 2007; Bihao *et al.*, 2012)** the expression of such gene at the correct time is the cause of a fatal cell death. The generation of tomato plants with delayed ripening **(Fraser *et al.*, 2002)** or application of the Cre/*lox*P marker-free self-excision strategy **(Moravčíková *et al.*, 2008; Roy *et al.*, 2008; Wang *et al.*, 2011).**
The last approach relies on the bacterial Cre/*lox*P recombination system that consists of the *cre* recombinase gene and two 34 bp *lox*P sites. The Cre recombinase excises any DNA sequence that is flanked on both sites by the *lox*P sequences, if they are oriented in a direct repeat **(Gilbertson, 2003)**. In the self-excision strategy, the *lox*P embedded DNA (floxed DNA) comprises both the *cre* and the selectable marker genes as a part of the same T-DNA. The *cre* recombinase could be driven by the inducible or developmentally regulated promoters. On activation, the Cre removes its own gene sequence as well as the marker gene. By using inducible promoters, the *cre* recombinase can be activated by the heat shock **(Fladung and Becker, 2010)** and chemically with β-estradiol **(Qiu et al., 2010)** or salicid acid **(Ma *et al.*, 2008)** as inducers. A more sophisticated approach includes self-excision controlled by the tissue specific promoters **(Mlynárová *et al.*, 2006; Bai *et al.*, 2008; Moravčíková *et al.*, 2008; Kopertekh *et al.*, 2009; Chong-Pérez *et al.*, 2013)**. The expression of the *cre* gene is targeted to certain time of plant development. However, the ectopic activity of such promoter causes premature excision of the selectable marker gene. Thus, regeneration under selectable pressure becomes toxic not only for non-transgenic but also for transgenic cells. The tissue-specific promoters without any ectopic activities are highly desired.
In this work we utilised the Cre/*lox*P recombination system to study the activity of the *Arabidopsis DLL* promoter under water stress treatment. If the stress triggers the activity of the *DLL*, the Cre recombinase will remove the *lox*P embedded DNA. The advantage of such strategy is the activity of the *DLL* can be recorded without any time-monitoring. In the *Arabidopsis*, the *DLL* drives a specific *AtOE16-S* gene expressed during seeds and pollen development **(Drea *et al.*, 2006)**. The *in silico* analyses of the promoter sequence using the PLACE database **(www.PLACE.com)** predicted the *DLL* promoter may be involved in response to abiotic stress.
To study the activity of the *DLL* under osmotic and drought stress conditions, the T-DNA containing the Cre/*lox*P self-excision recombination cassette was introduced into tobacco genome via *A. tumefaciens* LBA 4404. The expression of the *cre* gene was regulated by the activity of the *DLL* promoter. The inducibility of the *DLL* promoter in transgenic tobacco plants under water stresses was evaluated.

MATERIAL AND METHODS

Bacteria and vector construct

The binary vector pZP6 constructed previously **(Polóniová *et al.*, 2012)** was introduced into the strain *Agrobacterium tumefaciens* AGL-0. The T-DNA of the plasmid pZP6 carried the β-glucuronidase (*gus*) gene under control of the *dCaMV35S* promoter and one pair of the *lox*P sites that flanked the intron-containing *cre* recombinase (*cre*^{INT}) gene driven by the *DLL* promoter from *Arabidopsis* **(Drea *et al.*, 2006)** and the selectable neomycin phosphotransferase (*npt*II) gene. Bacterial cells were grown in Luria and Bertani (LB) medium **(Sambrook *et al.*, 1989)** containing 25 mg.l^{-1} rifampicin and 50 mg.l^{-1} kanamycin.

Plant material and transformation

Tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) was transformed with *A. tumefaciens* AGL-0/pZP6 using leaf discs transformation protocol described by **Horsch *et al.* (1985)**. The transformed tissue was selected on the medium with 100 mg.l^{-1} kanamycin. Each of selected (10) transgenic T_0/pZP6 plants were vegetatively multiplied into 5 clones. The 3 clones of each T_0 plant were

maintained under *in vitro* conditions. The rest 2 clones of each T_0 plant were transferred to the soil and cultivated in the greenhouse.

GUS assay

The activity of the *gus* gene were quantified fluorimetrically according to **Mlynárová *et al.* (1994)**. The GUS activity was expressed in pmol of methylumbelliferone released per min per µg of soluble proteins. The concentration of proteins was determined as described by **Bradford (1976)**.

PCR analyses

Genomic DNA was isolated from tobacco leaves using the DNeasy Plant Mini Kit (Qiagen, Manchester, UK). Internal PCR primers for detection of the *gus* gene were P1 (5´- GTT CCT GAT TAA CCA CAA ACC -3´) and P2 (5´- TGC ACA CTG ATA CTC TTC A -3´), for detection of the *nptII* gene were P3 (5´- ATG GGT CAC GAC GAG ATC ATC -3´) and P4 (5´- GAT GGA TTG CAC GCA GGT TCT -3´).
The excision event was verified using the primers P5 (5´- AAG AAT TCG AGC TCT GTA CC-3´) and P6 (5´-TCA GTG TGC ATG GCT GGA TA-3´).
The PCR reactions were carried out in 50 µl mixture containing 100 – 200 ng of DNA template, 15 pmol of each primer, 200 µM dNTPs, 1 × PCR buffer and 1 unit of Taq DNA polymerase (Finnzymes, Vantaa, Finland). The first PCR step of 94 ^oC for 4 min was followed by 30 cycles of 94 ^oC for 45 s, 62 ^oC for 45 s and 72 ^oC for 2 min. The last step was performed at 72 ^oC for 10 min.

Water stress treatment

The osmotic stress experiments were performed under *in vitro* conditions by transferring of 3 identical clones of each T_0/ZP6 plantlets lacking of roots to the glass test-tubes containing 2.5 ml liquid MS medium **(Murashige and Skoog, 1962)**. Following 5 days, the plantlets were divided into 3 groups. To the first and second groups, 2.5 ml of the liquid MS media supplemented with mannitol or PEG to the final concentration of 100 mmol.l^{-1} mannitol or 10 % PEG (w/v) (respectively) was added. To the third control group, 2.5 ml of the liquid MS media was added. The plantlets were subjected to the osmotic stress for 24 hours.
For drought stress experiments two identical clones per each (10) T_0/pZP6 plants were grown in soil. Following 30 days, the plants were divided into 2 groups. One group was watered as before but in the second group the watering was withheld. After 10 days, the third fully expanded leaf from the top of each stressed and non-stressed plants were collected.

Detection of lipid peroxidation

The levels of generated malondialdehyde (MDA) as product of lipid peroxidation were measured according to **Dhindsa and Matowe (1981)**. The concentration of MDA was determined in its unit equivalent using a molar extinction coefficient 155 x 10^5 mmol.l^{-1}

Qualitative detection of H_2O_2

Hydrogen peroxide was detected histochemically directly on the leaf tissue using solution of 1 mg.ml^{-1} (pH 3.8) 3,3'-diaminobenzidine-HCL (DAB-HCL) according to **Thordal-Christensen *et al.* (1997)**. Controls and treated leaf explants were placed into DAB-HCl and incubated for 3 hours in the dark. Afterwards explants were washed in distilled water and photographed.

RESULTS AND DISCUSSION

In this work, the activity of the tissue specific promoter *DLL* from *Arabidopsis* under water stress treatment was investigated. In origin plant, the corresponding *AtOE16-S* is a specific gene with an activity in maturation phase in seeds and pollen grains **(Drea *et al.*, 2006)**. A search for *cis*-element prediction using the online service of PLACE database identified four ACGT-core elements belonging to the G-box family of ABA-responsive elements (ABREs). They are found in the promoters of many genes expressed during dehydration **(Nakabayashi *et al.*, 2005)**. Due to *in silico* analyses we assumed the *DLL* promoter maybe involved in response to abiotic stress.
To study the activity of the *DLL* under water stress conditions, the T-DNA of the binary vector pZP6 (Fig. 1a) containing Cre/*lox*P self-excision recombination cassette was introduced into tobacco genome via *A. tumefaciens* LBA 4404. The expression of the *cre* gene was regulated by the activity of the *DLL* promoter. On activity of the *DLL*, the Cre/*lox*P cassette is removed.
Transgenic nature of regenerated T_0/ZP6 plants was firstly proved by histochemical (data not shown) and flourimerical (Figure 2) GUS assays. PCR analyses with the primer set P1/P2 confirmed the presence of the 742 bp corresponding to the *gus* gene in the genomes of analysed T_0 plants. An example of PCR analyses is given in Figure 2.
To find out whether the activity of the *DLL* promoter is regulated by water stress, selected (10) T_0/ZP6 plants were exposed to osmotic and drought stress treatments. The inducibility of the *DLL* promoter was monitored by PCR. The primer set P5/P6 was designed to amplify a 653 bp fragment confirming the excision of the Cre/*lox*P cassette (Figure 1b) because of the activity of the *DLL* promoter. In case the stress treatment did not induced the *DLL,* a 716 bp P5´-P6´ as well as 552 bp P3-P4 fragments are amplified (Figure 1a).
The ten *in vitro* grown T_0 plantlets were subjected to osmotic stress by 10 % PEG and 100 mmol.l^{-1} mannitol (individually) as inducers. The plantlets growing under the same but non-stressed conditions were used as a control. The applied concentration of inducers resulted in peroxidation of lipids in the plant cell membranes and generation of stress molecule H_2O_2 (Figure 4). However, PCR revealed amplifying the P5´-P6´ and the P3-P4 fragments in all stressed T_0 plants. Besides, no P5-P6 fragment was detected (Figure 3a, b). Thus, despite the obvious stress symptoms, the *DLL* activity remained stable.
In the drought stress *in vivo* experiments, the watering was withheld 10 days. To detect the activity of the *DLL*, plants were subjected to PCR analyses using the primer sets P3/P4 and P5/P6. The P5´-P6´ and the P3-P4 PCR products were detected in all stressed T_0 plants. No P5-P6 fragment was amplified (Figure 3). Similar to the results from the osmotic stresses, the *DLL* promoter was not induced under given drought stress conditions.
Intactness in the activity of the *DLL* to such abiotic stress treatments makes this promoter attractive for plant biotechnology. The precise spatial and temporal control of a gene expression is highly requested. For example, the cruciferin C (CRUC) gene from *Arabidospis* is considered as an embryo-specific **(Becerra *et al.*, 2006)**. However, the ectopic activity of the *CRUC* promoter was observed during regeneration of transformed tobacco cells. The application of the Cre/*lox*P self-excision marker-free strategy controlled by the *CRUC* promoter in tobacco resulted in unwanted premature excision of the selectable marker gene during *in vitro* regeneration **(Moravčíková *et al.*, 2008)**.

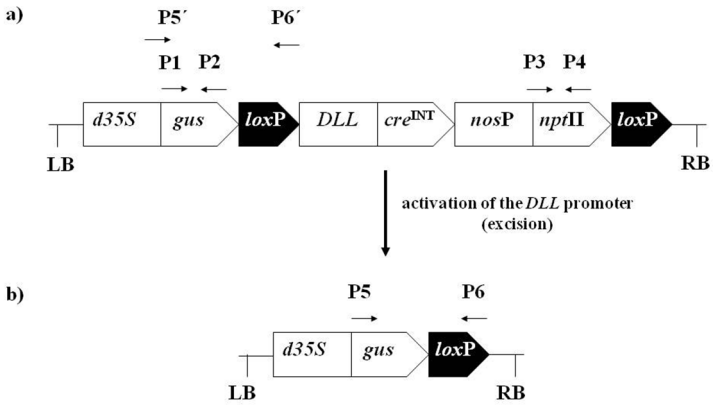

Figure 1 T-DNA structure of plant binary vector pZP6. **a)** The T-DNA consists of the reporter gene (*gus*) under control of the double *CaMV 35S* promoter (*dCaMV35S*), the intron-containing *cre* recombinase gene (*cre*) driven by the *Arabidopsis* promoter (*DLL*) and the neomycin phosphotransferase gene (*npt*II) regulated by the *nos* promoter. All genes are terminated by the *nos* terminator. Black arrows indicate position and orientation of the *lox*P sites. The sets of primers used for PCR analyses are indicated as P1/P2, P3/P4, P5/P6. In the case of no excision using the primers P5/P6, the P5´-P6´ fragment will be amplified. **b)** The T-DNA configuration generated after excision of the *lox*P embedded DNA. Other abbreviations used: RB, LB – right and left borders of T-DNA.

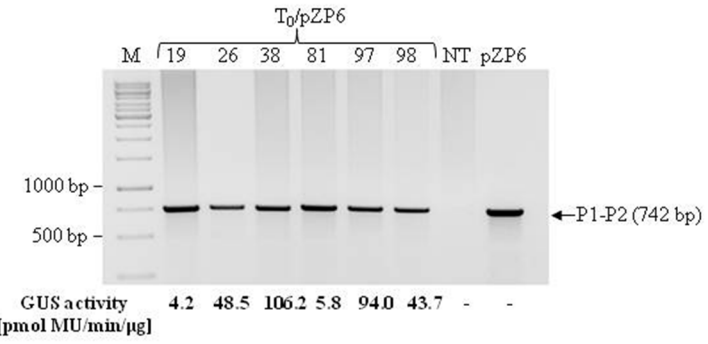

Figure 2 Photograph of the ethidium bromide-stained 1 % agarose gel carrying PCR fragments obtained on T_0 plants. PCR results with the primers P1/P2 that amplified an internal 742 bp fragment of the *gus* gene. The lane M contains 1 kb DNA ladder (Fermentas), the lanes 19 – 98 represent PCR products of T_0 plants, NT – non-transformed tobacco plant, pZP6 – plasmid pZP6 used for plant transformation. For each T_0 plants the value of GUS activity is given.

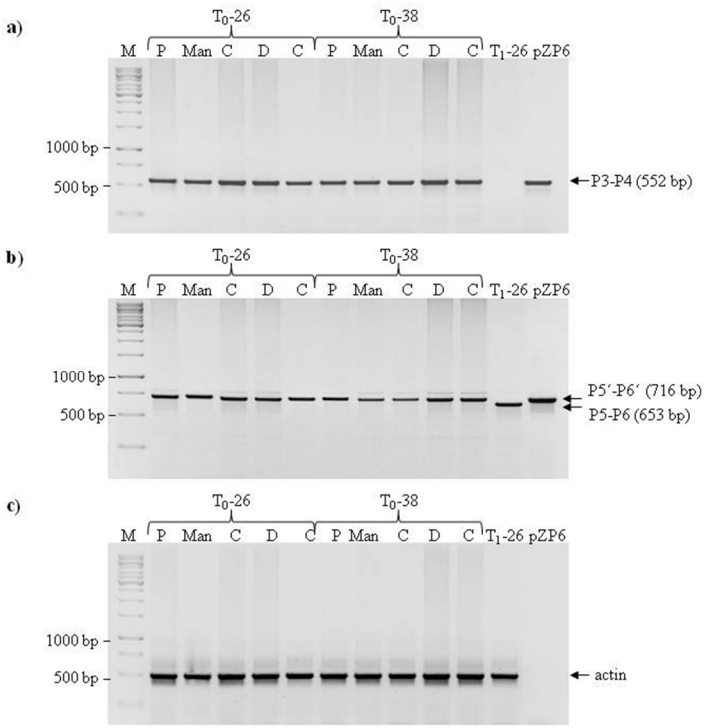

Figure 3 Photographs of ethidium bromide-stained 1 % agarose gels with PCR products obtained on transgenic tobacco $T_0$26 and $T_0$38 plants undregoing water stress. **a)** PCR results with the primers P3/P4 that amplified an internal 552 bp fragment of *npt*II gene. **b)** PCR results with the primers P5/P6 that amplified fragment 653 bp corresponding to the sequence generated after recombination event. In the case of no excision using the primers P5/P6, the P5´-P6´ 716 bp fragment is obtained. **c)** PCR products amplified with the actin primers. The lane M contains 1 kb DNA ladder (Fermentas). Other abbreviations used: P – 10 % PEG, Man – 100 nM mannitol, C – non-stressed control, D – drought, T_1-26 – progeny of the T_0-26 plant in which excision occurred during pollen and embryo development, pZP6 – plasmid pZP6 used for plant transformation.

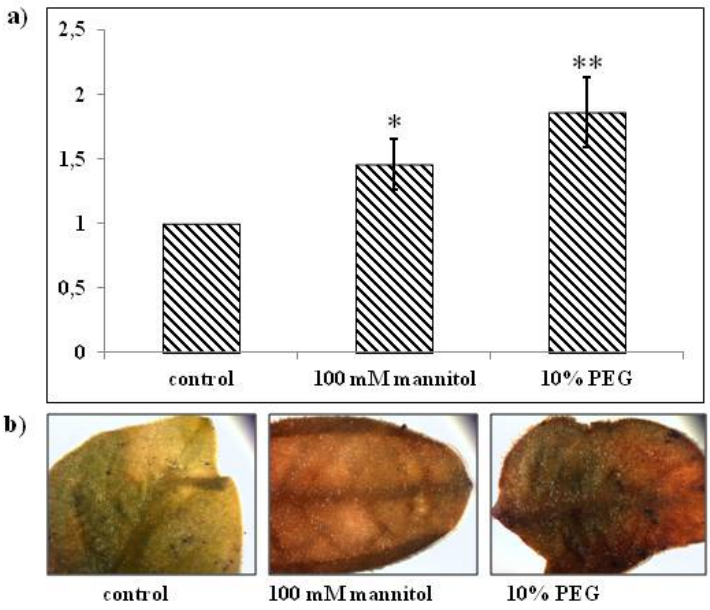

Figure 4 Indication on ongoing stress in tobacco T_0 plants. **a)** Relative content of malonyldiadelhyde in tobacco leaves exposed to 100 mM mannitol or 10 % PEG. Bars indicate ± standard error of mean values of 10 T_0/ZP6 plants. The data from analyses are significant as * and ** for $P < 0.01$ and for $P < 0.001$, respectively (Student´s *t*-test) **b)** Histochemical detection of stress molecules (H_2O_2) in tobacco leaves of T_0-26/pZP6 exposed to 100 mM mannitol or 10% PEG. Control – non-stressed plant.

CONCLUSION

With help of the Cre/*lox*P recombination system, the inducibility of the *Arabidopsis DLL* promoter under osmotic and drought stress conditions was investigated. For this, the T-DNA containing Cre/*lox*P self-excision recombination system controlled by the *DLL* promoter was introduced into tobacco genome. On activity of the *DLL* the Cre recombinase was expected to remove Cre/*lox*P cassette. The PCR analyses did not reveal any activity of the *DLL* promoter under given abiotic stress conditions. Our results point out the *DLL* as the promoter with precise control of the gene expression with wide usability in plant biotechnology.

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