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PREPARATION OF PLANT VECTOR CONSTRUCT CONTAINING DEHYDRIN GENE At2g21490

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ABSTRACT

This work is focused on preparation of a plant transformation vector pDH2 containing the expression unit of the dehydrin gene At2g21490 under a control of the constitutive *dCaMV 35S* promoter and the selectable marker neomycin phosphotransferase gene controlled by the *nos* promoter. The gene At2g21490 is classified as a dehydrin of the type K_2SY_2. The gene was isolated from *Arabidopsis thaliana* by PCR approach. The plasmid pDH2 was transformed into the strain *Agrobacterium tumefaciens* LBA 4404. The stability of the plasmid pDH2 in *Agrobacterium* has been verified by restriction analysis after re-transformation of the pDH2 into *Escherichia coli*.

Keywords: *Agrobacterium tumefaciens*, *Arabidopsis thaliana*, At2g21490, binary vector, dehydrins

INTRODUCTION

Due to the changes in the environment, plants are increasingly exposed to different types of abiotic stresses such as drought, salinity, low temperature or the presence of heavy metals. Among the proteins that are significantly accumulated during the stress caused by dehydration, osmoticum and/or a low temperature are LEA proteins ("Late embryogenesis abudant proteins") (**Amara *et al.*, 2014**). They are hydrophilic molecules whose role probably is to protect biomolecules and cell membranes (**Candat *et al.*, 2014**). Dehydrins (Pfam PF00257) are one of the most characterized group of LEA proteins. The LEPdb database currently contains over 380 records on dehydrins from various plant species and their physicochemical properties. Most of dehydrins are associated mainly with the process of embryogenesis, but they can also be accumulated in vegetative parts during the plant lifecycle. Generally, dehydrins are enriched with glycine and lysine residues, but they lack cysteine and tryptophan (**Allagulova *et al.*, 2003**). The characteristic feature of all dehydrins is conservative sequences denominated as K-, S- and Y-segmets which are their structural attribute. Five different subclasses of dehydrins are defined on the basis of the conserved segments: Y_nSK_n, SK_n, K_n, Y_nK_n, and K_n (**Close, 1997**). The presence of K-segment is obligate for all groups of dehydrins.

Dehydrins are considered as multifunctional proteins that can bind to macromolecules such as nucleic acid, have a protective role, can bind free metals, and can act as chaperones or antioxidants (**Rorat *et al.* 2004; Hara, 2010**). In term of the structure and the presence of characteristic protein domains, they are divided into several groups. So far, 10 dehydrin genes were identified in *Arabidopsis* (**Hundertmark and Hincha, 2008**). All of these genes contain histidine rich regions (His). Since the structures His-X3-His and His-His show strong affinity to metals (**Hara *et al.*, 2005**), it was supposed they can reduce various types of cellular damage during metal stress. The regions rich in His may play a role in buffering and/or may serve as sensors of the levels of metals (**Hara, 2010**) or could be involved in the reduction of "reactive oxygen species" (ROS) (**Hara *et al.*, 2013**).

The *Arabidopis* gene At2g21490 is classified as a dehydrin of the type K_2SY_2. It is expressed in late stages of seed formation and completely absent in vegetative tissues. The gene possesses 14 His residues, two His-X3-His and one His-His sequences (**Hara, 2010**).

In this work we prepared a plant transformation vector pDH2 containing the gene At2g21490 under control of the constitutive double *dCaMV 35S* promoter. The gene At2g21490 was isolated from *Arabidopsis thaliana* by PCR approach. The binary vector pDH2 was transformed into the strain *Agrobacterium tumefaciens* LBA 4404. The stability of the plasmid pDH2 in *Agrobacterium* has been verified by restriction analysis after re-transformation of the pDH2 into *Escherichia coli*.

MATERIAL AND METHODS

Izolation of the gene At2g21490

The genomic DNA from *Arabidpopsis thaliana* was isolated according to the **Chen et al. (1992)**. The sequence of the gene At2g21490 was amplified using the combination of the primers P1 (5´-GGTAATAATACCATGGGGGATTTGAG-3´) and P2 (5´-GCTAGCAAAATACAGTTCCTTC-3´). The PCR reaction was carried out in 25 µl mixture containing 100 – 200 ng of DNA template, 20 pmol of each primer (forward, reverse), 200 µM dNTPs, 1x PCR buffer and 1 unit of *Taq* DNA polymerase. The first PCR step of 95˚C for 4 minutes was followed by 35 cycles: 95˚C – 45 s; 63˚C – 45 s and 72˚C for 2 minutes. The last step was performed at 72˚C for 10 minutes. The PCR product was isolated from the gel using QIAquick Gel Extraction Kit (Qiagen). The identity of the PCR product was confirmed by sequencing.

Plant vector construct

The PCR product was ligated into the plasmid pGEm-T® Easy (Promega) to yield pZM1. Subsequently, a 1037 bp *Eco*RI-*Eco*RI fragment from pZM1 was ligated into *Eco*RI-*Eco*RI-digested plasmid pBSK+ to create pZM2. The plasmid pZM3 was created by cloning of *dCaMV35S* as *Hind*III-*Nco*I fragment from plasmid pBS4 and 762 bp *Nco*I-*Cla*I fragment from pZM9 into the vector pUN (**Vaculkova *et al.*, 2007**). The plant transformation vector pDH2 was obtained by ligation of *dCaMV35S/DH2/polyA* as *Eco*RI-*Xba*I fragment into *Eco*RI-*Xba*I-digested binary vector pBINPlus (**Van Engelen, 1995**). The binary vector pDH2 was introduced into *Agrobacterium tumefaciens* strain LBA 4404 using method „triparental matting" (**Matzke and Matzke**, 1986).

Stability of the plasmid pDH2 in *A. tumefaciens*

The stability was verified using restriction analysis after isolation of the plasmid pDH2 from *A. tumefaciens* and re-transformation into *E. coli*.

RESULTS AND DISCUSSION

To study the function of the dehydrin gene At2g21490 in tolerance to selected abiotic stresses using transgenosis, the plant transformation vector pDH2 was prepared. The T-DNA of the pDH2 contained the gene At2g21490 under control of constitutive *dCaMV 35S* promoter and the selectable neomycin phosphotransferase gene (*nptII*) under control of the *nos* promoter. The cloning strategy is given in Figure 1.

The specific primers P1/P2 were designed to amplify a PCR product P1-P2 of a size 1,037 kb that contained the sequence *DH2/polyA*. The plasmid pZM1 was created by ligation of the PCR product into the cloning vector pGEm-T® Easy. The identity of the amplified sequence was confirmed by sequencing. The sequence was compared with the sequence of the gene At2g21490 using the programe Clustal Omega (Figure 2).

The PCR product was ligated as *Eco*RI-*Eco*RI fragment of the plasmid pZM1 into the vector pBSK+ to yield plasmid pZM2. The identity of the inserted fragment was verified by restriction analysis (data not shown).

The next step of the cloning strategy was ligation of the sequence *DH2/polyA* from the pZM2 and the sequence of the *dCaMV 35S* promoter into the cloning vector pUN. Subsequently, the sequence of the expression unit *dCaMV 35S/DH2/polyA* was ligated into the binary vector pBinPlus. This vector contains the selectable *nptII* gene encoding resistance to antibiotic kanamycin. The identity of the plasmid pDH2 was confirmed by restriction analyses. In Figure 3a the position of restriction enzymes used in the analyses is given. The results of restriction analyses are in Figure 3b.

Based on our previous experiences with instability of some sequences in bacteria (**Vaculkova *et al.*, 2007**), the stability of the sequence of the T-DNA region was analysed using restriction analyses after re-transformation of the plasmid pDH2 from *A. tumefaciens* into *E.coli*. After retransformation, totally, 30 clones were isolated and subjected to restriction analyses. Restriction profiles of all analysed clones corresponded with expected (Figure 4).

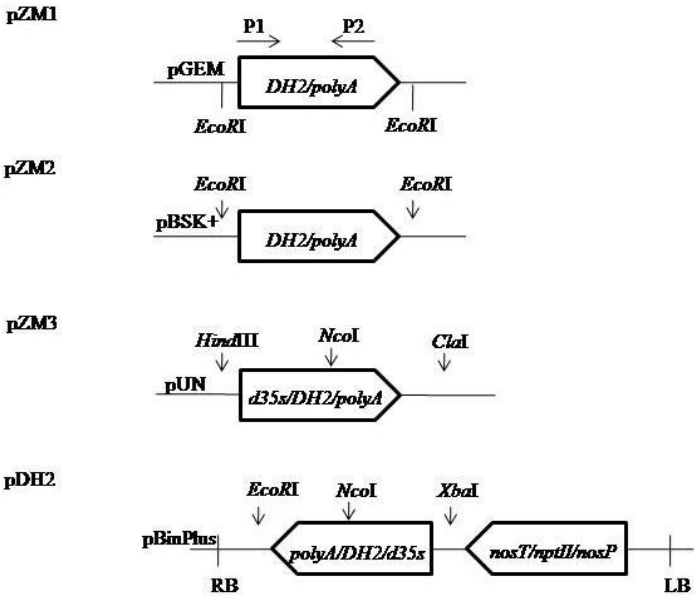

Figure 1 Cloning strategy. The arrows indicate restriction enzymes used in individual cloning steps. *d35s* – double *CaMV 35S* promoter, *dh2*- dehydrin gene, *polyA* – poly sequence of gene DH2, *nosT* – nos terminator, *npt*II – neomycin phosphotransferase gene, *nosP* – nos promoter.

Figure 2 The alignment of the sequence P1-P2 with the sequence of the gene At2g21490. Alignment was generated using the CLUSTAL OMEGA program. Nucleotides which are conserved in the sequences aligned are marked by asterisks. Dashes show sequence that is important for polyadenylation of mRNA. The arrows outline the position of primers P1 and P2. The sequence of the primers P1 and P2 are in the boxes.

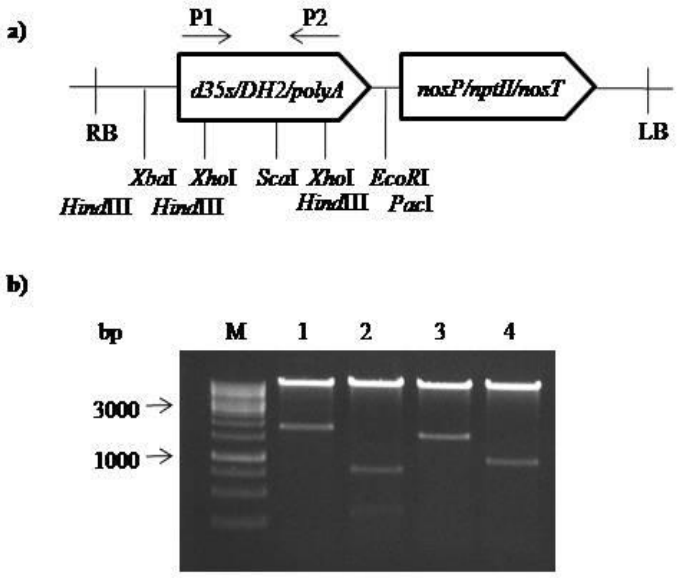

Figure 3 Restriction map and restriction analyses of the T-DNA-pDH2. **a)** T-DNA structure of the pDH2. The primers used for PCR analyses are indicated as P1, P2. The position of restriction enzymes used in restriction analyses is indicated **b)** Photograph of the ethidium bromide-stained 1 % agarose gel carrying in lane M - 1 kb DNA marker (Fermentas), lane 1 – pDH2 / *Eco*RI+*Xba*I, lane 2 – pDH2/ *Xho*I, lane 3 – pDH2 / *Hind*III, lane 4 – pDH2/ *Asc*I+*Sca*I.

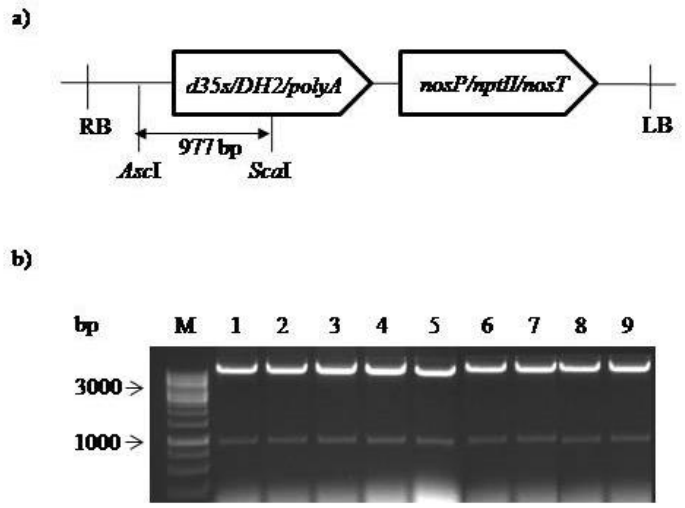

Figure 4 Restriction analyses of the T-DNA-pDH2 after re-transformation of plasmid into *E. coli*. **a)** The plasmid pDH2 with the position of restriction enzymes used in analyses. **b)** Photograph of the ethidium bromide-stained 1 % agarose gel carrying in lane M - 1 Kb DNA marker (Fermentas); lanes 1-9 – pDH2/*Asc*I + *Sca*I .

CONCLUSION

Using the techniques of recombinant DNA a plant transformation vector pDH2 was prepared. The T-DNA region of the pDH2 contained *Arabidopsis* gene At2g21490 under control of the constitutive *dCaMV 35S* promoter and selectable marker *npt*II gene providing resistance to the antibiotic kanamycin. The binary vector pDH2 has been shown to be stable in *A. tumefaciens* LBA 4404. In future, *A. tumefaciens* LBA 4404 carrying the plant transformation vector pDH2 will be used for transformation of tobacco leaf explants.

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