JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

THE PRODUCTION OF TRANSGENIC TOBACCO PLANTS OVEREXPRESSING OAK DEHYDRIN GENE

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https://doi.org/10.55251/jmbfs.9225

ARTICLE INFO

Received 20. 7. 2022
Revised 30. 9. 2022
Accepted 3. 10. 2022
Published 21. 12. 2022

Regular article

OPEN ACCESS

ABSTRACT

This work is focused on the production of transgenic tobacco plants overexpressing the oak dehydrin gene AY607707.1. Transgenic tobacco plants were generated via *Agrobacterium*-mediated transformation. The T-DNA of the plant transformation vector pMK contained the dehydrin gene fused to the constitutive double dCaMV35S promoter and the selectable neomycin phosphotransferase gene. The sequence of the dehydrin gene was isolated from *Quercus robur* by PCR approach and cloned. The constructed binary vector pMK was introduced into *Agrobacterium tumefaciens* LBA4404 and used in the transformation experiments. Transgenic plants were generated with an efficiency of 32.7%. PCR analyses confirmed the transgenic nature of regenerated T_0 plants. The expression of the oak dehydrin gene was proved by RT-PCR and quantified by qPCR analyses.

Keywords: *Agrobacterium tumefaciens*, dehydrins, *Nicotiana tabacum L.*, *Quercus robur*, transgenic plants

INTRODUCTION

Dehydrins (PF00257) belong to a group of LEA proteins (**Jaspard *et al.*, 2012**). They are thermostable and highly hydrophilic proteins that are associated mainly with later stages of plant embryogenesis (**Amara *et al.*, 2014)**. Besides that, many studies indicate a role of dehydrins in plant stress tolerance. They are accumulated under low temperature, water deficiency, osmotic or heavy metal stress (**Close, 1997**; **Hanin *et al.*, 2011**; **Kosova *et al.*, 2019; Riyazuddin *et al.*, 2021)**. Dehydrins are defined by the presence of a lysine-rich motif called the K-segment (EKKGIMDKIKEKLPG). The K-segment can form an amphiphilic α-helix that may contribute to the stabilisation of the proteins and cellular membranes. Some dehydrins contain also motifs called Y- and S- segments. The Y-segment could be a likely place for binding nucleotides. The S-segment consists of serine residues and acts as a site of phosphorylation. Based on the presence of these conserved segments, dehydrins are classified into five main subclasses: Kn, SKn, KnS, YnKn and YnSKn **(Battaglia *et al.*, 2008)**.

In general, dehydrins are considered multifunctional proteins, but not all dehydrins perform all functions. Some functions are common to many dehydrins, while some may be specific, e.g. ability to bind nucleic acids (**Boddington *et al.*, 2019**). They can act as cryoprotectants, chaperones, or ROS scavengers. Dehydrins can bind free metal ions, phospholipids, or proteins (**Hara, 2010; Eriksson *et al.*, 2016; Hara *et al.*, 2016**). The roles of dehydrins in plant development and stress tolerance have been studied either in their native organism (**Eriksson *et al.*, 2016; Hara *et al.*, 2016)** or using a system of transgenic plants (**Xu *et al.*, 2008; Liu *et al.*, 2019**).

Despite intensive research on dehydrin genes over the past 20 years, dehydrins in woody plants are relatively little explored. Trees located in the temperate zone need to survive the cold season, so they must tolerate the shortening of daylight and low temperatures (**Welling et al., 2004**). Thus, dehydrins in woody plants are mainly associated with freezing tolerance and dormancy.

In the genus *Quercus*, dehydrins have been studied in the species *Q. robur*, *Q. petraea*, *Q. ilex*, or *Q. cerris*. In the species *Q. robur*, three dehydrins QrDhn1 (YSK_3-type), QrDhn2 (K_4-type), and QrDhn3 (K_3-type) have been identified so far (**Šunderlíková *et al.*, 2009**). Northern blot analysis revealed, that these dehydrins respond to altered water and osmotic conditions.

In this work, we prepared a plant transformation vector pMK and used it for *Agrobacterium*-mediated genetic modification of tobacco. The plant transformation vector pMK contained in its T-DNA an expression cassette consisting of the oak dehydrin gene Dhn3 under the control of a constitutive double dCaMV 35S promoter. The sequence of the oak dehydrin gene (AY607707.1) was isolated by PCR approach. Transgenic plants overexpressing the oak dehydrin were regenerated under the selection pressure of kanamycin. The transgenic nature of regenerated plants was confirmed by molecular analyses.

MATERIAL AND METHODS

Plant transformation vector preparation

The sequence of the oak dehydrin gene (AY607707.1) was isolated by PCR approach using the primer set P1/P2 (Table 1). The genomic DNA was extracted from the leaves of *Quercus robur* according to the protocol by **Chen *et al.* (1992)**. The PCR reaction mixture (25 µl) consisted of 100-200 ng DNA, 200 µM dNTPs, 20 pmol of the primers, 1× PCR buffer and 1 unit of FIREPol® DNA polymerase (Solis BioDyne). The first PCR step of 95˚C for 4 minutes was followed by 35 cycles: 95˚C 45 s; 64˚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) and commercially sequenced.

The PCR product was ligated into the cloning vector pJET1.2 (CloneJet PCR Cloning Kit, Thermo Fisher Scientific). Then, the sequence Dhn3 as a 350 bp *Xho*I-*Xba*I fragment from the plasmid pJET1.2-Dhn3 and the *polyA* sequence as a 268 bp *Xba*I-*Hind*III fragment from the plasmid pRT100 **(Töpfer *et al.;* 1987)** were ligated into the cloning vector pSK+. The plant transformation vector pMK (Figure 1A) was prepared by the ligation of the *Dhn3/polyA* sequence as a 648 bp *Eco*RI-*Nco*I fragment and the sequence d*CaMV*35S as a 736 bp *Nco*I-*Hind*III fragment into the *Eco*RI a *Hind*III digested plasmid pBinPlus (**Van Engelen, 1995).** The plasmid pMK was transformed into *Agrobacterium tumefaciens* LBA 4404 and used in the transformation experiments.

Tobacco transformation and regeneration

Tobacco plants (*Nicotiana tabacum* L. cv. Petit Havana SR1) were cultivated in *in vitro* conditions at 20 ± 2ºC with a day length of 16 h under 50 µE m^{-2} s^{-1} light intensity. Leaf segments of six-weeks-old *in vitro* tobacco plants were transformed *via Agrobacterium*-mediated transformation as was described by **Polóniová *et al.* (2015)**. Transformed tobacco cells were regenerated under the selection pressure of kanamycin (50 mg/L). The transformation efficiency was evaluated as the number of shoots that were rooted in the presence of kanamycin with respect to the total number of explants used in the experiment.

Molecular characterisation of transgenic plants

DNA was isolated from the leaves of tobacco plants using a DNeasy® Plant Mini Kit (QIAGEN). PCR analyses were performed with the primers P1/P2, P3/P4, and P5/P6 that were designed to confirm the presence of the dehydrin Dhn3 and the selectable *npt*II genes; and the actin as a housekeeping gene, respectively (Table 1). The PCR mixture and PCR reaction conditions are given above.
Total RNA was isolated from leaves of tobacco plants using RNeasy® Plant Mini Kit (QIAGEN). RNA was reverse transcribed using Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific).
RT-PCR analyses were realised with the primer sets P7/P8 and P5/P6 to confirm the presence of genes Dhn3 and actin, respectively (Table 1). The PCR reaction mixture (25 µl) consisted of 20 ng cDNA, 200 µM dNTPs, 20 pmol of the primers, 1× PCR buffer and 1 unit of FIREPol® DNA polymerase (Solis BioDyne). The PCR reaction conditions are given above.
The qPCR analyses were realised with the primer sets P9/P10 and P5/P6 that amplified fragments corresponding to the genes Dhn3 and actin, respectively (Table 1). The analyses were performed using a LightCycler® Nano System (ROCHE). The qPCR reaction conditions were according to the manufacturer´s instructions (Luminaris HiGreen qPCR Master Mix, ThermoFisher Scientific). The expression of the dehydrin gene Dhn3 was quantified using the ΔCt method.

Table 1 Primers used in PCR analyses, expected sizes and purpose

Primer code	Primer	Sequence (5´- 3´)	Expected size [bp]	Purpose
P1	Forward	CCATGGAAAATGTCGCATTACCAAAACCAGC	346	Isolation of the Dhn3 gene PCR
P2	Reverse	CACCTAGTGGAGTCCAGGAATCTT		
P3	Forward	GATGGATTGCACGCAGGTTCT	552	*npt*II gene (internal fragment) PCR
P4	Reverse	ATGGGTCACGACGAGATCATC		
P5	Forward	GCACCTCTCAACCCAAAG	102	actin (housekeeping gene) PCR, RT-PCR, qPCR
P6	Reverse	GGAAAGGACAGCCTGAATAG		
P7	Forward	GCCCAGCTATCTGTCACTTCAT	251	Dhn3 gene (internal fragment) RT-PCR
P8	Reverse	CTTCTCCTTGATCTTCTCCATCACTC		
P9	Forward	GCCCAGCTATCTGTCACTTCAT	111	Dhn3 gene (internal fragment) qPCR
P10	Reverse	CCTCCTCCTGATGATGATGTTGG		

RESULTS AND DISCUSSION

Dehydrins belong to a group of hydrophilic proteins that are associated with the stress response in plants. Their main role is thought to be to prevent protein inactivation and aggregation. Dehydrins are characterised by the presence of conservative regions called K-, S- and Y-segments. Based on their structure, they can be divided into five types YnSKn, YnKn, SKn, Kn and KnS, which to some extent determine their function (**Battaglia *et al*., 2008**). In this study, we isolated genomic DNA from the leaves of *Quercus robur* and used it as a template for PCR to amplify the sequence of the oak dehydrin gene (Dhn3) (Figure 1C). The isolated intronless sequence Dhn3 was of a size 346 bp. The deduced Dhn3 protein contains 112 amino-acid residues with a molecular weight of about 12.3 kDa and a predicted isoelectric point of 9.66. The dehydrin Dhn3 contains three repeats of K segments and structurally belongs to the Kn-type of dehydrins.

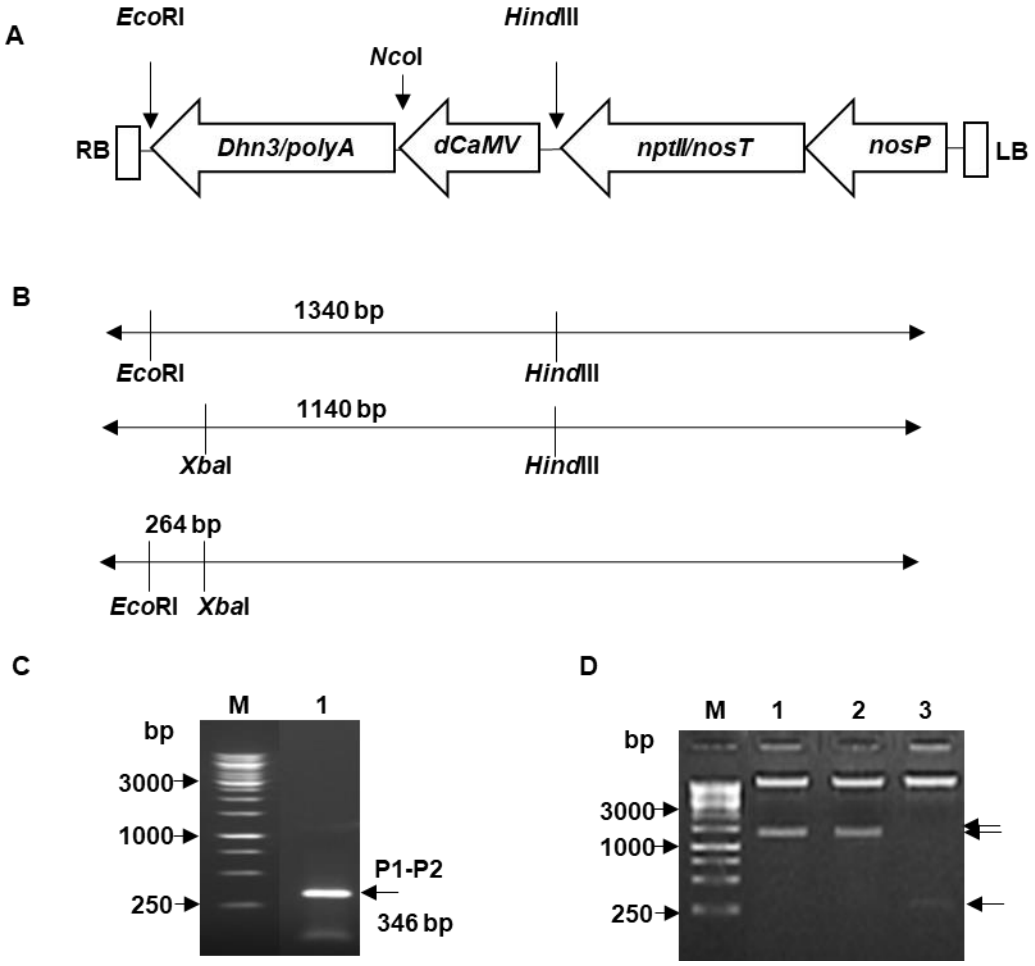

Figure 1 The T-DNA of the plant transformation vector pMK (**A**); Restriction map of the T-DNA-pMK (**B**); PCR product of the size a 346 bp (Lane 1) corresponding to the sequence of the gene Dhn3 in ethidium bromide stained 1% agarose gel (**C**); Restriction analyses of the plasmid pMK2 (**D**), M - 1kb DNA ladder (ThermoFisher Scientific), Lane 1 - pMK/*Eco*RI+*Hind*III, Lane 2 - pMK/*Xba*I+*Hind*III, Lane 3 - pMK/*Eco*RI+*Xba*I.

The plant transformation vector pMK (Figure 1A) was created by the introduction of the expression cassette consisting of the Dhn3 gene fused to the constitutive d*CaMV* 35S promoter as a 736 bp d*CaMV 35S/Dhn3/polyA* fragment into the binary vector pBinPlus. The plasmid pBinPlus is a derivate of the binary vector pBin19 (**Bevan *et al*., 1984**). The T-DNA of the plasmid pBinPlus contains a selectable marker *npt*II gene that is placed at the left T-DNA border (LB). Given that the transfer of T-DNA is polar (**Lee and Gelvin, 2008**), the location of the *npt*II gene at the LB border increases the likelihood of regenerating transgenic plants with an intact T-DNA. The identity of the plasmid pMK was confirmed by restriction analyses (Figure 1B, Figure 1D).

Transgenic tobacco plants were generated via *Agrobacterium*-mediated transformation. Leaf explants (Figure 2A) were infected with bacterial inoculum *A. tumefaciens* LBA4404/pMK. Since the pMK-T-DNA contains the *npt*II marker gene, which encodes resistance to antibiotics such as kanamycin or neomycin, transformed cells were regenerated under the selection pressure of kanamycin (50 mg/L). We used a total of 104 explants for transformation experiments. The first calli appeared after two weeks (Figure 2B). The first shoots began to form 4-6 weeks after transformation (Figures 2C). We obtained 72 shoots, of which 34 (47.2%) rooted in the presence of kanamycin (50 mg/L). These (T_0) plants were considered as putative transgenic. We achieved a transformation efficiency of 32.7%, which is comparable to the results in other studies, where **Moravčíková *et al.* (2008)** reports a transformation efficiency of 39.0%, **Hlozáková *et al.* (2014)** 40.4% or **Polóniová *et al.* (2015)** 57.6%.

Putative transgenic T_0 plants (Figure 2D) were subjected to molecular analyses to confirm their transgenic nature (Figure 3). The presence of the dehydrin Dhn3 and the marker *npt*II genes were confirmed with the primer sets P1/P2 and P3/P4 that were designed to amplify the fragments of the sizes 346 bp and 552 bp, respectively (Figure 3A). The fragments of the expected sizes were identified in all (12) analysed T_0 plants. We did not observe the PCR products in wild-type tobacco plants. RT-PCR analyses were used to detect the expression of the dehydrin Dhn3 gene in transgenic T_0 plants. Analyses were performed with the primers P7/P8 that were designed to amplify a 251 bp fragment (Figure 3B). The PCR product of the expected size was detected in all (12) analysed T_0 plants. The relative expression levels of the dehydrin Dhn3 in transgenic tobacco were quantified by qPCR with the primer set P9/P10. As shown in Figure 3C, the relative expression level of the Dhn3 was variable depending on the transgenic T_0 line.

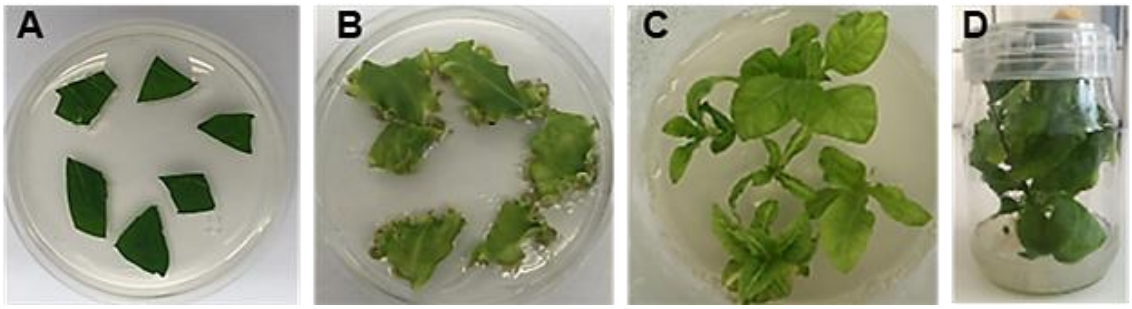

Figure 2 Regeneration of transformed tobacco cells under the selection pressure of 50 mg/L kanamycin. (**A**) Infected leaf explants on regeneration media; (**B**) Callus formation; (**C**) Shoot elongation; (**D**) Transgenic tobacco rooted in the presence of kanamycin.

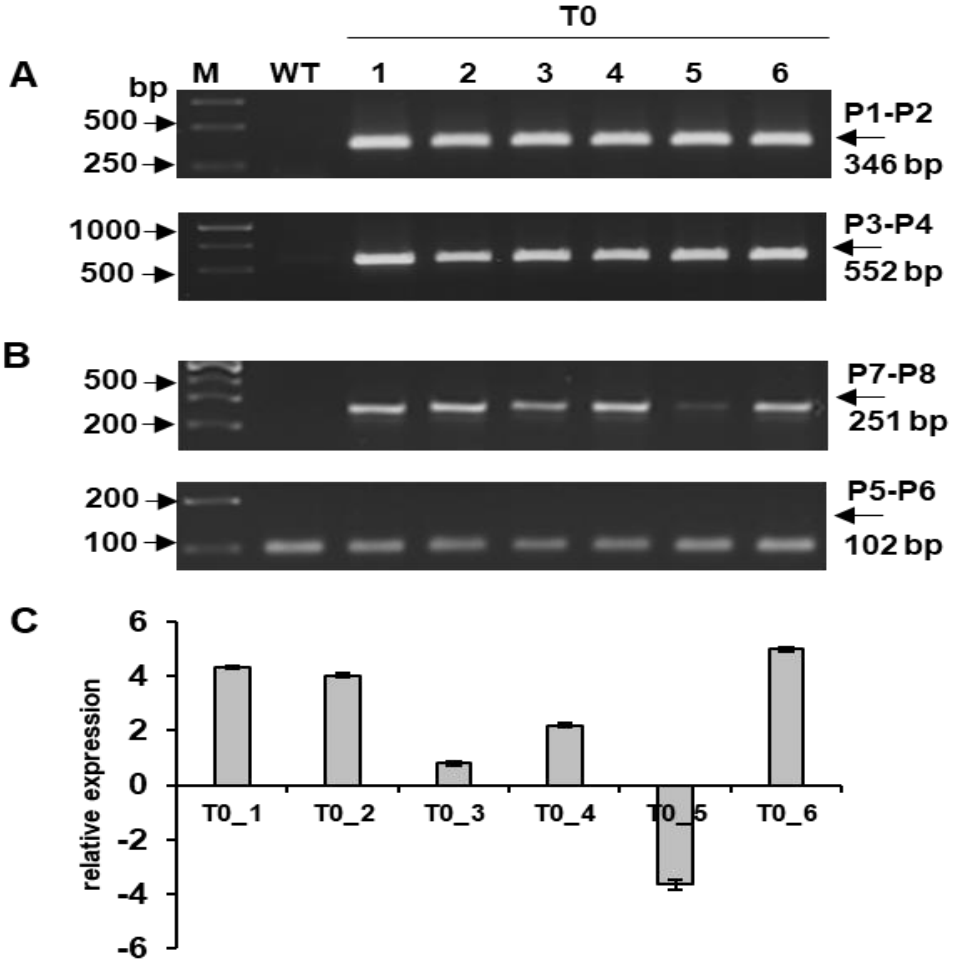

Figure 3 Molecular analyses of transgenic T_0 plants. (**A**) PCR analyses performed on genomic DNAs from transgenic T_0 and wild-type (WT) tobacco plants with the specific primers for the genes Dhn3 (P1/P2) and *npt*II (P3/P4). (**B**) RT-PCR analyses performed on cDNAs from transgenic T_0 and wild-type (WT) tobacco plants with the specific primers for the genes Dhn3 (P7/P8) and actin (P5/P6). (**C**) Quantification of the expression level of the gene Dhn3 in transgenic T_0 plants. The qPCR analyses were performed with the specific primers P9/P10 (Dhn3) and P5/P6 (actin as a housekeeping gene). Lane M – DNA ladder (ThermoFisher Scientific), WT non-transgenic tobacco plants, Lanes 1-6 transgenicT_0 plants.

CONCLUSION

Transgenic tobacco plants overexpressing oak dehydrin gene were generated via *Agrobacterium*-mediated transformation. The T-DNA of the plant transformation vector pMK contained the oak dehydrin gene under the control of the constitutive d*CaMV* 35S promoter and the selectable marker *npt*II gene. The sequence of the oak dehydrin gene Dhn3 from the *Quercus robur* was isolated based on the PCR approach and cloned. The constructed plasmid pMK was transformed into *A. tumefaciens* LBA 4404 and used in the transformation experiments. Transgenic plants overexpressing the dehydrin gene Dhn3 were generated with an efficiency of 32.7%. Transgenic nature of regenerated T_0 plants was confirmed by PCR analyses. The expression of the Dhn3 gene was proved by RT-PCR and quantified by qPCR analyses. Transgenic plants with the highest expression levels of the transgene will be used in further experiments focused on the effect of overexpression of the oak dehydrin gene on plant stress tolerance.

Acknowledgements: This work was supported by the grants from the Slovak Grant Agency VEGA 1/0525/20, the institutional resources within the University Grant Agency FPV-08-22 and the Operational Programme Integrated Infrastructure for the project IMTS 313011V344, co-financed by the European Regional Development Fund.

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