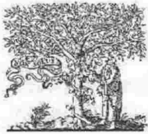
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South African Journal of Botany 73 (2007) 505–511

SOUTH AFRICAN JOURNAL OF BOTANY

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Transformed tobacco plants with increased tolerance to drought

J. Gubiš [a,*], R. Vaňková [b], V. Červená [a], M. Draguňová [c], M. Hudcovicová [a], H. Lichtnerová [c], T. Dokupil [c], Z. Jureková [d]

[a] Slovak Agricultural Research Centre, Research Institute of Plant Production, Bratislavská cesta 122, 921 68 Piešťany, Slovak Republic
[b] Laboratory of Hormonal Regulations in Plants, Institute of Experimental Botany ASCR v.v.i., Rozvojová 263, 165 02 Praha 6, Czech Republic
[c] Laboratory of Explantate Cultures, Horticulture and Landscape Engineering Faculty, Slovak University of Agriculture, Tulipánová 7, 946 76 Nitra, Slovak Republic
[d] Department of Ecology, Faculty of European Studies and Regional Development, Slovak University of Agriculture, Mariánska 10, 949 76 Nitra, Slovak Republic

Received 8 November 2006; received in revised form 29 March 2007; accepted 31 March 2007

Abstract

P5CSF129A cDNA and the *npt*II marker gene were used for tobacco (*Nicotiana tabacum* L. cv. Bel B and cv. M51) transformation via *Agrobacterium tumefaciens* strain LBA4404. Twenty transformed tobacco plants were obtained after transformation of leaf discs. Presence of the transgene was confirmed by polymerase chain reaction (PCR) analysis. Physiological responses to water stress were compared in transgenic and wild-type tobacco plants. Transgenic plants of both cultivars accumulated high levels of free proline. They did not exhibit dry mass relocation or chlorophyll content reduction. Neither precocious senescence, nor leaf necrosis or morphological changes were observed in control and stress conditions (RWC decrease by 7–8%). Transgenic plants with elevated accumulation of osmoprotectants seem to be better adapted to water stress, providing a perspective for future research of stress effects that have a principle role in the functional activity of plants. This study confirmed *P5CSF129A* to be a candidate gene in crop engineering for enhanced water stress tolerance.
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Keywords: *Nicotiana tabacum* L.; Pigment; Proline; Relative water content; Stress

1. Introduction

Plants are often exposed to various adverse environmental stresses such as drought, salinity, high and low temperatures. The organisms respond to the change of environmental conditions by activation of enzyme reaction products which contribute to the re-establishment of homeostasis. During water stress, lower and higher plants accumulate osmoprotectants, such as glycinebetaine and proline (Gorham et al., 1985; Delauney and Verma, 1993). The typical levels of naturally accumulated osmoprotectants are 5–50 μmol/g of fresh weight (~6–60 mM on a plant water basis), maxima are reached during exposure to osmotic stress (Rhodes and Hanson, 1993; Bohnert et al., 1995).

The effectiveness of osmotic adjustment by osmoprotectants was proved using transgenic plants (Bohnert and Jensen, 1996; Nuccio et al., 1999; Rontein et al., 2002), which accumulated higher concentrations of proline, glycinebetaine, or pinitol (Kishor et al., 1995; Zhang et al., 1995; Sheveleva et al., 1997; Takabe et al., 1998). An alternative strategy is regulation of water permeability through water channels, which may also modify the sensitivity to water stress (Aharon et al., 2003).

Overexpression of the Δ^1-pyrroline-5carboxylate synthetase gene (*P5CS*) encoding the first enzyme of the proline biosynthesis pathway was reported to enhance proline production in transgenic plants (Kishor et al., 1995). The level of proline can be increased 2-fold (Hong et al., 2000) provided that the feedback inhibition by proline is removed. This was done by Zhang et al. (1995), who used the site directed mutagenesis in order to replace Phe at position 129 by Ala in *Vigna aconitifolia P5CS* gene (*P5CSF129A*).

Recently, successful attempts have been made to transfer genes involved in stress tolerance into plants. The resulting

* Corresponding author.
E-mail address: gubis@vurv.sk (J. Gubiš).

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doi:10.1016/j.sajb.2007.03.011

transgenic plants were reported to exhibit enhanced tolerance to water stress and other stresses (Xu et al., 1996; Hsieh et al., 2002; Bhattacharya et al., 2004; Chandra Babu et al., 2004). However, information about physiological reactions of modified plants is still very limited.

Tobacco is an important model plant that has been extensively used for the study of genetic, physiological and metabolic processes (Elleuch et al., 2001), ranging from tissue culture establishment, via floral growth and development (Mandel et al., 1992) to the evaluation of the impact of environmental pollution (Monciardini et al., 1998). The aim of our study was transformation of tobacco leaf discs with a gene improving drought tolerance and evaluation of the stress response and adaptation ability of the resulting transformed tobacco.

2. Materials and methods

2.1. Plant material: in vitro explants

Seeds of *Nicotiana tabacum* L. cultivars Bel B and M51, respectively, were surface-decontaminated by shaking in 70% ethanol for 1 min and in 4% sodium hypochlorite for 10 min. Subsequently they were rinsed four times with sterile distilled water. The seeds were then allowed to germinate in glass containers with 25 cm^3 of MS-based medium (Murashige and Skoog, 1962). The cultures were initially kept in the dark at 27±1 °C for 7 days and then maintained under a photoperiod of 16 h illumination with a light intensity of 50 μmol m^{-2} s^{-1}, with day/night temperatures of 25 °C/20 °C.

2.2. Transformation via Agrobacterium tumefaciens

A plasmid (pBI-P5CSF129A) containing mutagenized *V. aconitifolia P5CSF129A* cDNA (Zhang et al., 1995) under the control of the cauliflower mosaic virus 35S promoter, and neomycine phosphotransferase (*npt*II) marker gene was used for tobacco transformation via *Agrobacterium tumefaciens* strain LBA4404. For tobacco transformation, 8-week-old leaves were cut into 50×50 mm discs, inoculated with bacterial suspension at OD_{600}=0.6 for 15 min and blotted dry with sterile filter paper to remove excess bacterial suspension. The inoculated leaf discs were transferred to MS-based medium containing 1 mg dm^{-3} BAP, and kept in the dark for 3 days. After co-cultivation, the transformed leaf discs were transferred to shoot regeneration medium supplemented with 1 mg dm^{-3} BAP, 100 mg dm^{-3} kanamycin, 250 mg dm^{-3} cefotaxime and 250 mg dm^{-3} carbenicillin under the photoperiod as described above. Eight weeks later, when regenerated plantlets reached 20–30 mm in height, they were cut off and placed on a selective rooting medium (MS-based medium+100 mg dm^{-3} kanamycin). Rooted plantlets were transferred to *ex vitro* conditions.

2.3. DNA extraction and PCR analysis

For molecular analysis of transgenic tobacco, plant genomic DNA was extracted from 1.5 g fresh plant tissue according to Dellaporta et al. (1983). PCR analysis was performed using specific primers to detect *P5CSF129A* gene (forward 5′-GTGAAGA-TAACGCCTGGAGC-3′and reverse 5′- GTTTTCCACCAACAT-TATCTG-3′) amplifying an amplicon of 468 bp and *npt*II gene (forward 5′-CAGACAATCGGCTGCTCTGAT-3′and reverse 5′-TGCGAT GTTTCGCTTGGTGGT-3′) amplifying an amplicon of 330 bp. The PCR procedure was performed as follows. DNA (1 ml) was added to a final volume 15 ml with 0.3 ml of each forward and reverse primers in concentration 0.2 μM, 1× PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl, 1.5 nM $MgCl_2$), 0.2 mM of dNTP, 0.8 units of Taq DNA polymerase, and 25 ng of DNA. PCR with the *P5CSF129A* gene was performed in GeneAmp® PCR System 9700 (Applied Biosystems) under the following conditions: at 94 °C for 4 min, 30 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and extra extension at 72 °C for 8 min. PCR with the *npt*II gene was performed at 94 °C for 5 min, 30 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 45 s, and extra extension at 72 °C for 8 min. Electrophoretic detection of PCR products was performed in 1.4% agarose gels stained with ethidium bromide.

2.4. Histochemical analysis

The expression of *GUS* gene was detected by histochemical staining according to Jefferson et al. (1987). Leaf segments of transgenic plants were immersed in 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-GlcA) solution at 37 °C overnight and rinsed with 70% ethanol. The expression of the *GUS* gene was inspected microscopically. The transient expression efficiency of the *GUS* gene was calculated as the number of positive segments compared to the number of segments tested.

2.5. Stress treatment

Rooted plantlets were acclimatised for 4 weeks and then transplanted to pots with soil. The plants were grown in greenhouse at defined temperature (25–30 °C) and relative humidity (65–85%) in summer. Plants at the stage of 5 leaves (wild type cv. M51 and cv. Bel B as well as the transformed ones) were divided into 2 groups: control (70% water content in soil) and plants stressed by drought (40% water content in soil). After the 40% water content in soil was reached, this level of water stress was constantly maintained. The first analysis of samples was made before inducing stress, and then on days 12 and 18 after treatment. The following parameters were quantified: fresh (FW) and dry (DW) weights of plants, relative water content, content of free proline and content of pigments.

2.6. Proline determination

The proline content was determined in leaf samples (1 g FW) according to Bates et al. (1973) by measuring the quantity of the coloured product of proline reaction with ninhydric acid. The absorbance was read at 519 nm using a Spectroquant® Spectrophotometer NOVA 400 (Merck Ltd.). The proline concentration was determined from a standard curve and calculated on a fresh weight basis (μmol proline g^{-1} FW).

2.7. Determination of relative water content (RWC)

Leaves collected for determination of the relative water content ($RWC = [(FW - DW)/(FW_{saturated} - DW)] \times 100\%$) were of similar physiological age as those collected for proline determination. The leaves were re-cut in water, and kept in distilled water in a closed glass flask at room temperature (21 °C). The FW of the leaves was determined before and after incubating for 5 h at room temperature. Leaves were then dried for 48 h at 65 °C to determine DW.

2.8. Determination of pigment contents

The content of chlorophyll *a*, *b* and carotenoids in leaves was determined after extraction in 80% acetone at 20 °C. Absorption was measured at 663 nm, 645 nm and 440 nm using a Spectroquant® Spectrophotometer NOVA 400 (Merck Ltd.). The leaf samples were of similar physiological age as those collected for proline and RWC determination.

2.9. Statistics

The data presented in this paper is the mean from two independent experiments. All data were analysed for significance ($P \leq 0.05$) by ANOVA with mean separation by Duncan's test using statistical software SPSS (13.0).

3. Results and discussion

The aim of our study was to obtain regenerated tobacco shoots transformed with proline biosynthetic gene in order to improve plant tolerance to stresses. A total of 40 explants of tobacco cv. Bel B and cv. M51, respectively, were transformed with *A. tumefaciens* carrying plasmid with gene *P5CSF129A*. Five putatively transformed shoots of cv. Bel B and 15 of cv. M51 were cultivated on selective medium (Fig. 1a). Regeneration frequency of cv. Bel B and cv. M51 was 50% (1.33 shoots per regenerated explant) and 52.5% (1.4 shoots per regenerated explant), respectively. All plants transplanted to soil survived acclimatisation. No regeneration of control (untransformed) explants was observed on regeneration medium supplemented with 100 mg dm^{-3} kanamycin (data not shown).

After selection and multiplication under *in vitro* conditions, the regenerated shoots were tested for the presence of introduced genes using PCR analysis. Also the rooted shoots transplanted to soil (Fig. 1b, c) were subjected to PCR analysis which confirmed the presence of both — *npt*II and *P5CSF129A* genes. The presence of both genes was confirmed in all tested samples originating from *in vitro* cultures as well as in *ex vitro* acclimatised plants before and after the stress treatment (Fig. 2). The *in vitro* rooted transformants were also tested using the histochemical GUS assay. All the regenerated shoots proved to be GUS-positive (Fig. 3).

The synthesis and accumulation of free proline was already established in the *in vitro* conditions by means of quantification of free proline content in the organ culture. Explants of transgenic cv. M51 accumulated free proline in quantities of 8.05 μmol/g FW, in comparison with 0.37 μmol/g FW detected in wild-type M51. The transgenic cv. Bel B accumulated 12.75 μmol/g FW (0.34 μmol/g FW was detected in wild-type Bel B). A similar pattern of free proline accumulation was found in plants grown in a greenhouse (transformed ones in comparison with the wild-type), under both conditions — sufficiently supplied with water and subjected to water deficit. Significant differences ($P \leq 0.05$) in proline content were detected also among the genotypes used. On average, free proline accumulation in leaves of transformed tobacco plants in control conditions (70% water content in the soil) was almost 17-fold (cv. M51) and 22-fold (cv. Bel B) higher than in the wild-type lines. The free proline accumulation in leaves under stress conditions (40% water content in the soil) was found to be 14-fold (cv. M51) and 16-fold (cv. Bel B) higher when compared to stressed wild-type plants (Fig. 4). Under stress conditions, proline accumulates in higher quantities in leaves of cv. M51 than in the leaves of cv. Bel B.

Overproduction of proline in plants may increase the tolerance to abiotic stresses. Kishor et al. (1995) showed that

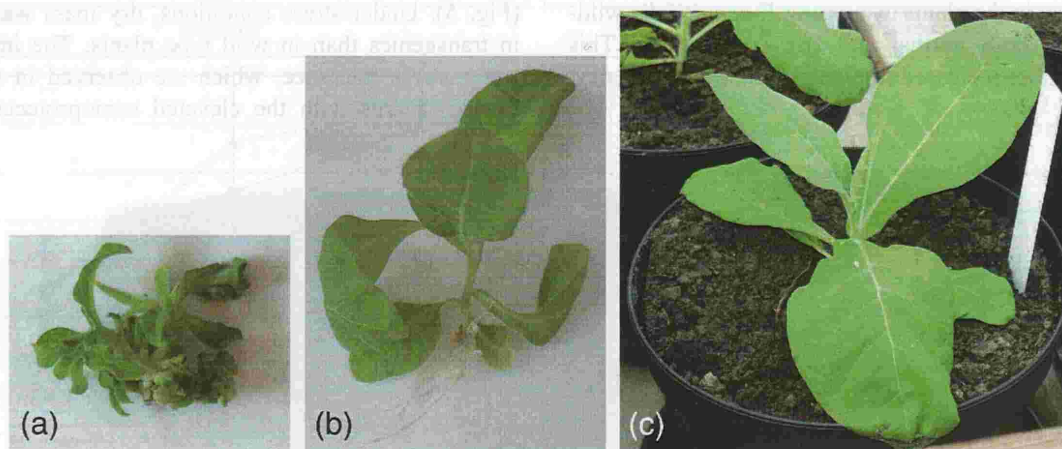

Fig. 1. Transgenic tobacco transformed with *A. tumefaciens* LBA4404 carrying the *npt*II and *P5CSF129A* genes from *V. aconitifolia*. (a) Shoot regeneration on leaf-discs of *N. tabacum* L. cv. M51, (b) regenerated shoot after rooting on selective medium, (c) L0 line of transgenic tobacco.

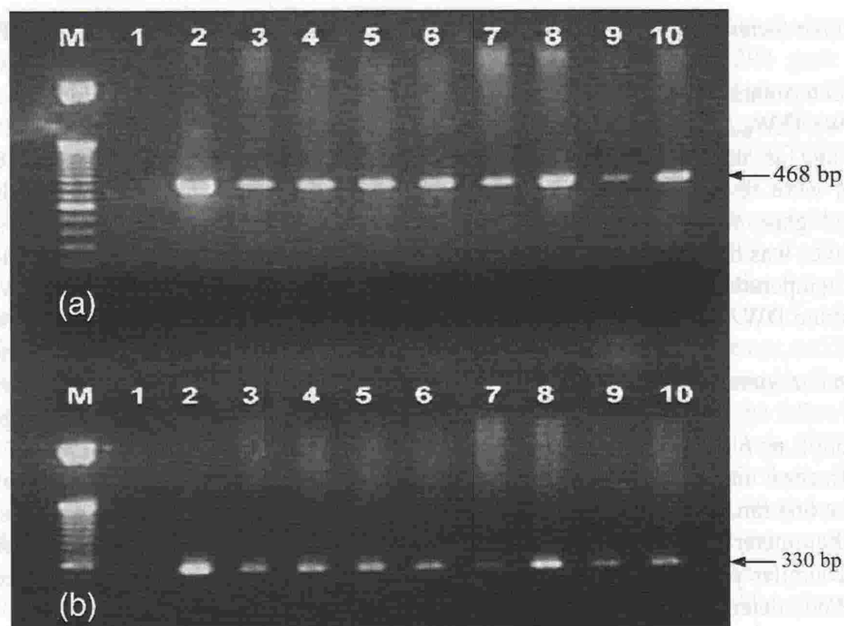

Fig. 2. Molecular analysis of L0 lines of transgenic tobacco. (A) PCR analysis using *P5CSF129A* gene primers, M: 50 bp ladder marker (Invitrogen Life Technologies, USA); lane 1: water as negative control; lane 2: DNA of pBI-P5CSF129A plasmid as positive control; lanes 3–6: transformed tobacco lines from *ex vitro* plants; lanes 7–10: transformed tobacco from *in vitro* conditions. (B) PCR analysis using *npt*II gene primers, M: 50 bp ladder marker (Invitrogen Life Technologies, USA); lane 1: water as negative control; lane 2: DNA of pBI-P5CSF129A plasmid as positive control; lanes 3–6: transformed tobacco lines from *ex vitro* plants; lanes 7–10: transformed tobacco from *in vitro*.

proline accumulation correlated with tolerance to drought and salinity stresses in plants. Transgenic plants of tobacco prepared by Kishor et al. (1995) synthesized 10- to 18-fold more proline than control plants. The osmotic potential of leaf sap from transgenic plants was less decreased under water stress conditions compared to those of control plants. Overproduction of proline also enhanced root biomass and flower development in transgenic plants under drought stress conditions. Accumulation of proline in plants under stress may offer multiple benefits to the cell. A role of proline in scavenging free radicals in cells exposed to salinity has been clearly shown by Hong et al. (2000). These authors also studied the proline biosynthesis regulation. They showed that removal of feedback inhibition in *P5CSF129A* resulted in a 2-fold increase in proline content compared with that in the plants expressing *V. aconitifolia* wild-type *P5CS* under both normal and stress conditions. This difference was further increased in plants treated with 200 mM NaCl.

Except the *P5CS* gene, other genes were exploited in tobacco transformation to improve plant tolerance to drought stress e.g. *TPS1* gene (Romero et al., 1997), *ots*A and *ots*B genes (Goddijn et al., 1997), *lp3* gene (Wang et al., 2003), *Asr1* gene (Kalifa et al., 2004), *BoRS1* gene (Tang et al., 2005), *AtTPS1* gene (Almeida et al., 2005), *CAP2* gene (Shukla et al., 2006) and *NtHSP70-1* drought-/ABA-inducible gene (Cho and Hong, 2006).

Physiological characterization of the studied genotypes revealed different abilities of biomass production. Cultivar Bel B had a slightly higher capability of dry mass production; however, it was more sensitive in stress conditions. Transformation did not have negative effects on the dynamics of dry mass production especially in cv. Bel B (Fig. 5). Under stress conditions, dry mass was reduced less in transgenics than in wild type plants. The improvement of water stress tolerance, which we observed in our transgenic tobacco plants with the elevated osmoprotectant content, is

Fig. 3. Histochemical GUS assay of a transformant. Blue precipitations represent the activity of the GUS gene.

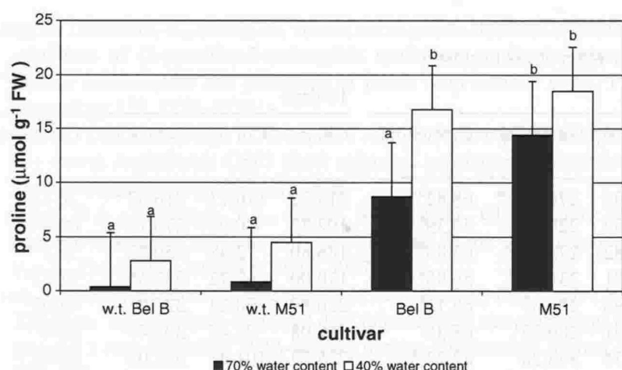

Fig. 4. Proline content in wild-type and transgenic tobacco leaves in control (soil water content 70% — black bars) and stress (soil water content 40% — white bars) conditions. Data represent the mean values from three offtakes ($n=6$). Duncan's test at 5% level of significance. The error bars show SE.

in accordance with the results of Delauney and Verma (1993).

Increase of abiotic stress tolerance in model plants and presumably in other crops over-expressing the *AtTPS1* gene (involved in trehalose biosynthesis in *Arabidopsis thaliana*) was suggested by Almeida et al. (2005), who observed higher germination rates under both osmotic and temperature stress in tobacco plants containing *AtTPS1* gene.

In this study, leaf relative water content (RWC) was also followed. The control plants had 86–94% of RWC. Cessation of water supply resulted in a slow decline of RWC. Two percent decline in the leaf RWC was determined in wild-type M51 as well as in transgenic Bel B after 12 days of water stress, whilst in transgenic M51 and wild-type Bel B the leaf RWC declined by 5%. After 6 more days of water stress, a decline by approximately 4–5% in leaf RWC in wild-type M51 and Bel B and 7–8% in transgenic plants Bel B and M51 was observed.

Exposure of the tobacco plants to water stress for 12 and 18 days resulted in the elevation of the content of Chl *a*, Chl *b*, total Chl and carotenoids (Table 1). Statistically significant differences ($P \leq 0.05$) in the total Chl and carotenoids were found between cultivars as well as during deficit progression. The content of total Chl and carotenoids was particularly affected by genotype. In wild-type cv. Bel B the total Chl and carotenoid contents increased by about 31 and 36%, respectively, after 12 days of water stress treatment. Increase in the total Chl and carotenoid contents continued to 44 and 34%, respectively, recorded after 18 days of water stress treatment. In transgenic cv. Bel B the total Chl and carotenoid contents increased by about 52 and 63%, respectively after 12 days of water stress treatment, whilst a 47 and 37% increase, respectively in the total Chl and carotenoid contents was recorded after 18 days of water stress treatment. In wild-type M51 total Chl and carotenoid contents increased by about 2 and 10%, respectively, after 12 days of water stress treatment. Eighteen days after the water stress treatment the content of both total Chl and carotenoids increased

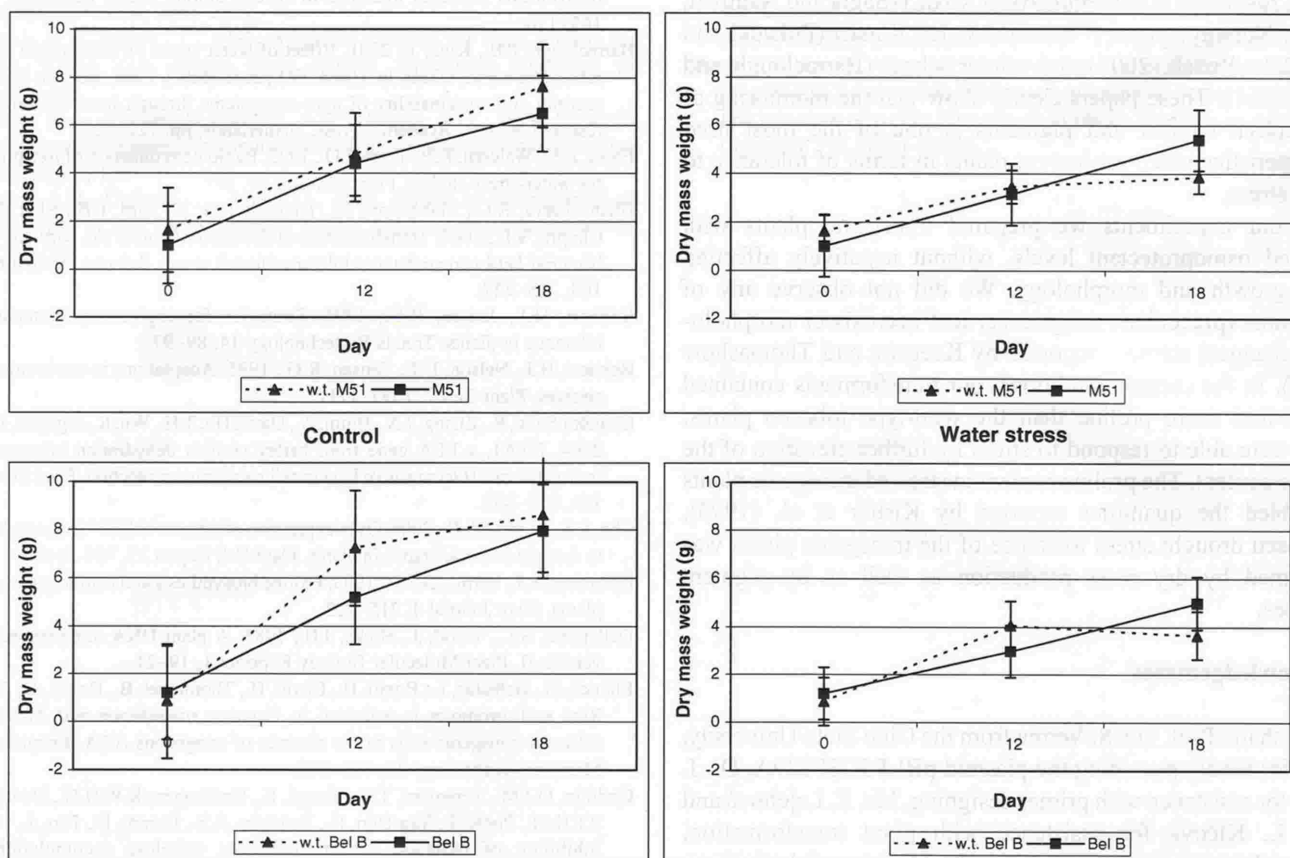

Fig. 5. The effect of water stress on dry mass accumulation in leaves of wild-type and transgenic tobacco plants at 0, 12 and 18 days of water stress treatment. The bars show standard errors.

Table 1
Photosynthetic pigment content [mg m^{-2} (FW)] in tobacco cvs., 0, 12 and 18 days after water stress treatment

Treatment		0 day				12 days				18 days			
Cultivar	Water content in soil (%)	Chl *a*	Chl *b*	Total Chl	Carotenoids	Chl *a*	Chl *b*	Total Chl	Carotenoids	Chl *a*	Chl *b*	Total Chl	Carotenoids
w.t. M51	40	198.38	73.13	271.45^{a}	62.58^{a}	198.68	77.94	276.55^{bc}*	68.82^{b}*	215.72	170.73	316.37^{cd}*	70.82^{d}
	70					165.55	63.32	228.81^{a}	54.39^{a}	192.87	80.98	273.78^{b}	60.89^{bc}
M51	40	200.49	74.14	274.57^{a}	62.00^{a}	200.16	73.82	273.91^{bc}	63.80^{ab}	186.80	72.48	259.22^{ab}	58.3^{b}*
	70					174.60	60.21	234.75^{ab}	56.95^{a}	170.00	67.72	237.65^{a}	51.51^{a}
w.t. Bel B	40	166.24	61.72	227.90^{a}	50.83^{a}	214.47	83.98	298.37^{c}	69.11^{b}	230.89	98.27	329.08^{cd}	68.03^{d}
	70					207.81	81.51	289.25^{c}	68.36^{b}	215.08	92.47	307.47^{c}	65.4^{cd}
Bel B	40	169.72	59.55	229.21^{a}	50.58^{a}	250.55	97.74	348.20^{d}	82.22^{c}*	235.73	101.41	337.05^{d}	69.49^{d}*
	70					218.02	93.85	311.79^{cd}	66.42^{b}	194.41	85.78	280.12^{b}	57.37^{b}

* Significantly different from control (70% water content) at $P \le 0.05$. Superscript letters mean significant difference at $P \le 0.05$.

by about 17 and 13%, respectively. Significant difference was also found in transgenic cv. M51, with the increase of carotenoid content by about 3% after 12 days of water stress treatment (Table 1). The effect of drought stress on chlorophyll content may differ in individual leaves depending on stress severity and duration as well as on plant tolerance (Taiz and Zeiger, 2006). Increase in chlorophyll content under drought and salinity stress was reported by Mäkelä et al. (2000) in tomato, Özmen et al. (2003) in barley and Michelozzi and Johnson (1995) in clones of *Eucalyptus grandii* × *E. robusta*. On the other side, the decrease in chlorophyll content during the water stress was reported in Indian rosewood (*Dalbergia sissoo* Roxb.) (Sagta and Nautiyal, 2002), Norway spruce (*Picea abies* (L.) Karst.) (Pukacki and Kamiñska-Rozek, 2005) and winter wheat (Barraclough and Kyte, 2001). These papers clearly show that the monitoring of chlorophyll content and pigments is one of the most used characteristics when evaluating plants in terms of tolerance to water stress.

In our experiments we prepared transgenic plants with elevated osmoprotectant levels, without negatively affecting plant growth and morphology. We did not observe any of symptoms (precocious senescence, leaf necrosis or morphological changes) recently reported by Keegstra and Thomashow (2002). In the control conditions, our transformants contained 17–22-fold more proline than the wild-type tobacco plants. They were able to respond to stress by further elevation of the proline content. The proline content in stressed transgenic plants resembled the quantities reported by Kishor et al. (1995). Increased drought stress tolerance of the transgenic plants was confirmed by dry mass production as well as by pigment analyses.

Acknowledgements

We thank Prof. D.P.S. Verma from the Ohio State University, USA for kindly providing the plasmid pBI-P5CSF129A, Dr. J. Kraic for assistance with primer designing, Ms. Z. Lajchová and Mrs. L. Klčová for assistance with plant transformation. Financial support was provided by the Ministry of Agriculture of the Slovak Republic, Grant No. 2003 SP 27/028 0D 01/028 0D 01, by Grant No. 1/9075/02 from the Scientific Grant Agency VEGA of the Ministry of Education, Slovakia, and by the Slovak Academy of Sciences.

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Edited by E Balazs