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Animal Anti–Apoptotic Genes Enhance Recovery from Drought Stress in Tobacco

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ABSTRACT

The anti–apoptotic nematode CED–9 gene and a 3' non–coding mRNA region of the human Bcl–2 gene, referred to as 161–1, enhanced resistance to drought and recovery after re–watering in transgenic *Nicotiana tabacum* L. CV Glurk plants. The effect of drought on plant functions was investigated by withholding water from pots for 6, 8, 10 and 13 days. Results showed that controls and transgenic tobacco did not differ among each other under well–watered conditions. Differences appeared however, on day 3 of water deprivation, where controls exhibited a significantly steeper decline in photosynthesis (A) compared to the transgenic lines. On day 10 of drought stress, losses due to respiration exceeded gains from photosynthesis in Glurk and G115 (vector control), but not in CED–9 and 161–1. Stomatal conductance (g_s) was generally higher in the transgenic when compared to controls, and there were no differences between controls and transgenic tobacco in relative water content. Significant differences were found among lines recovering after 13 days of water deprivation. CED–9 and 161–1 exhibited significantly higher values of A on day 17 than controls. Recovery of controls was significantly affected after prolonged periods of water stress; 84 and 82% of leaves in 161–1 and CED–9 recovered from drought, comparing to 70 and 67% in Glurk and G115, respectively. The most notable differences were observed in the number of plants flowering after re–watering, where 67% of 161–1 plants flowered compared to 33 and 30% of CED–9 and G115, respectively. Glurk had the lowest number of plants recovering, with only 17% of its individuals flowering after re–watering.

Key Words: Anti–apoptotic genes; Programmed cell death; *Nicotiana tabaccum;* Drought tolerance; Gas exchange

INTRODUCTION

There has been an increasing interest over the last few years in programmed cell death (PCD) or apoptosis due to its crucial role in the development and specialization of higher animal cells, tissue homeostasis, and disease (Adams & Cory, 1998). Programmed cell death is a physiological cell suicide of unwanted cells (Ellis *et al*., 1991). It can also be triggered in response to pathogen attack (Dickman *et al*., 2001). In plants, selective cell death, similar to apoptosis has been reported (Barlow, 1982; Watanabe *et al*., 2002) and was found to occur during vegetative and reproductive phases of plant development (Pennell & Lamb, 1997). The recent and first completion of the sequence of *Arabidopsis* genome did not show the presence of anti–apoptotic or cell death suppressor genes in plants (Lam *et al*., 2001). Anti–apoptotic genes that negatively regulate apoptosis, such as those expressing chicken Bcl–xl and nematode CED–9 genes were found to confer resistance to several necrotrophic fungal pathogens and to tomato spotted wilt virus in transgenic plants (Dickman *et al*., 2001). Bcl–xl and CED–9 were also found to prevent cell death induced by either UV radiation or paraquat treatment (Mitsuhara *et al*., 1999), to enhance resistance to salt, cold and wound stresses (Qiao *et al*., 2002) and to ameliorate the loss of turgor in water–stressed transgenic tobacco (Awada *et al*., 2003).

Anti–apoptotic genes have the potential to suppress cell death induced by a broad spectrum of stresses in plants as they do in animals. Anti–apoptotic genes are speculated to prevent cell death in a number of ways including: preventing the loss of mitochondria permeability under stresses (Qiao *et al*., 2002) and the subsequent release of cytochrome c that is known in some cases to activate death proteases termed caspases (Adams & Cory, 1998); maintaining the vacuole homeostasis, which is necessary for the survival of plants during drought (Dietz *et al*., 2001) and preventing oxygen–induced cell death by maintaining the homeostasis of chloroplast (Qiao *et al*., 2002).

Programmed cell death pathways are activated in animal cells in response to wide array of environmental stresses including rapid thermal and osmotic changes, toxins, pathogens, or damaging radiation. These types of stressors are associated with the disruption of mitochondrial and vacuole membrane permeability and the generation of reactive oxygen species (ROS). Several animal anti–apoptotic genes are associated with the mitigation of these stress–induced cell deaths, particularly the Bcl–2 family members (Gross *et al*., 1999) and thus appear to have overlapping stress–mitigating functions. To evaluate the potential conservation of cell survival pathways between plants and animals, we have generated and partially characterized tobacco plants expressing certain members of the Bcl–2 family. With the knowledge that water stressed plants develop relatively high levels of ROS (Price *et al*.,

1989; McKersie *et al.*, 1996; Oberschall *et al.*, 2000) and/or ROS indicators, including glutathione transferase/peroxidase (Bianchi *et al*., 2002), we hypothesized that plants with anti–apoptotic genes would have enhanced tolerance to and recovery from water deficit conditions by interfering with ROS mediated cell death.

Recently, Awada *et al.* (2003) have shown that anti–apoptotic genes ameliorated loss of turgor in transgenic tobacco transformed with the 3' non–coding region of the human Bcl–2, chicken Bcl–xl and nematode CED–9. The authors however did not examine whether this amelioration is mirrored with an improvement in recovery after re–watering. In the present study we used tobacco plants transformed the CED–9 gene and a 3' non–coding, Bcl–2 mRNA to determine whether expression of these genes enhances drought resistance and recovery from water stress in tobacco plants.

MATERIALS AND METHODS

Nicotiana tabacum L. cv Glurk plants were transformed as described by Dickman *et al*. (2001) with one of the two negative regulators of apoptosis: 1) human Bcl–2 mRNA sequence 725–1428 representing a 3' non–coding region of the gene and shown to confer resistance to certain pathogens (human Bcl–2 gene mRNA obtained from S. Korsmeyer, Dana–Faber Cancer Institute, Boston) (Yang & Korsmeyer, 1996), referred to as 161–1; 2) *C. elegans* CED–9 gene, which suppresses PCD in the nematode (obtained from H. Horvitz, Massachusetts Institute of Technology, Cambridge) (Hengartner & Horvitz, 1994). Glurk tobacco or Glurk tobacco plants transformed with G115 (the plasmid transformation vector containing β–glucuronidase) served as controls. All plants (wild type & transgenic) were similar in morphology and development.

The tobacco lines used were all from original transformants and were vegetatively propagated. In our initial studies (Dickman *et al*., 2001); we screened a minimum of three independent events per transgene and 10 lines per event, and specifically chose plants that expressed a low (1–2) copy number of insertions, as determined by DNA blots. Lines were also chosen based upon normal morphological features. There was a perfect (100%) correlation between phenotype and genotype when tested for gene expression and resistance to necrotrophic fungi. In the present study, we continued with a selected line from each genotype which was characterized for amelioration of loss of turgor with drought stress (Awada *et al*., 2003).

Four week–old tobacco plants were transferred to pots filled with Cornell Mix. Plants were grown in the greenhouse for 8 weeks under ambient light conditions at a relative humidity of 50% and at 26°:22°C day:night temperatures. Twenty four individuals were selected from each plant type (total of 96) with relatively identical height (75 cm). Individuals were distributed randomly between four plots, and pots were spaced at 30 cm to prevent shading. Soil moisture levels were monitored in all pots throughout the study using Time Domain Reflectometry (TDR) technology (TH_2O portable soil moisture meter, Dynamax Inc., Houston, TX) (Fig. 1). Soil moisture deficit was initiated by withholding water for 6, 8, 10 and 13 days in plots 1 through 4, respectively. Physiological and morphological measurements were taken on all plots every 2–3 days for 17 days to determine plant responses to drought and recovery after re–watering.

Maximum net photosynthesis (A, μmol m^{-2} s^{-1}) and stomatal conductance (g_s, mol m^{-2} s^{-1}) at light saturation were followed during water deprivation and after re–watering, using a portable photosynthetic system mounted with a LED light source (LI 6400–2B, LICOR Inc. Lincoln, NE). Measurements were conducted between 11:00 am and 2:00 pm, at an air temperature of 25–26°C, relative humidity of 45% and photosynthetic active radiation (PAR) equal to 1200 μmol m^{-2} s^{-1}. Relative water content (RWC, %) of the leaves [RWC=((fresh weight–dry weight)/(turgid weight–dry weight))*100] was determined on the same leaves used for gas exchange measurements. Percent of live leaves (green leaves with complete recovery after watering) per plant and number of plants flowering after re–watering was assessed.

Statistical analysis. Repeated measures analyses were used to evaluate plant responses. The data were analyzed using the Mixed Models Procedure in SAS (SAS Institute, 1996). Pairwise means comparisons were performed using the probability of difference (Pdiff statement in SAS, $P<0.05$) (Steel *et al.*, 1997). Orthogonal contrasts were performed for each of these analyses of variances: transformed tobacco vs. controls, among controls, and among transformed tobacco.

RESULTS

Responses to drought. The effect of drought on controls (Glurk & G115) and transgenic (CED–9 & 161–1) tobacco plants was investigated by withholding water from pots for 6, 8, 10 and 13 days before re–watering. Analysis of variance results (Table I) showed that under well watered conditions (the day water stress was initiated), A, g_s and RWC of the leaves did not differ among plant types. Plant responses to water deprivation were analyzed using orthogonal contrasts, trends among controls were similar. On day 3 of water deprivation, orthogonal contrasts (Table I) showed that A was significantly greater in transgenic plants (16 μmol m^{-2} s^{-1}) than in the controls (12 μmol m^{-2} s^{-1}) (Fig. 2). This indicates a significantly steeper decline in A in the controls compared to the transformed at the beginning of water deprivation. Photosynthesis continued to decline in all lines with the increase in drought stress, and approached zero on day 10 of water deprivation. During this period differences among lines were minimal, except on day 10, where losses due to respiration (negative A) exceeded gains from photosynthesis in Glurk and G115 (–0.2 μmol m^{-2} s^{-1}),

Table I. Analysis of variance and orthogonal contrasts results of photosynthesis (A), stomatal conductance (g_s) and relative water content (RWC) in controls (Glurk & G115) and transgenic (CED-9 and 161-1) tobacco plants under well watered conditions and 3, 6, 8, 10 and 13 days of water deprivation.

		A		g_s		RWC	
Source	**df**	**F**	**P**	**F**	**P**	**F**	**P**
Plant type (well watered)	3	0.68	0.5	0.22	0.88	0.52	0.73
Plant type (3 days of water deprivation)	3	1.34	0.28	0.76	0.52	1.02	0.41
CED-9 vs 161-1 † Glurk vs G115 (CED-9 161-1) vs (Glurk G115)	88	3.60	0.02				
(CED-9) vs (161-1 Glurk G115)	88			2.1	0.05		
Plant type (6 days of water deprivation)	3	1.03	0.40	0.74	0.53	0.84	0.63
(161-1) vs (CED-9 Glurk G115)				2.03	0.05		
Plant type (8 days of water deprivation)	3	0.05	0.98	0.40	0.75	0.95	0.43
Plant type (10 days of water deprivation)	3	0.59	0.62	0.25	0.85	0.52	0.55
(161-1) vs (CED-9 Glurk G115)	88	2.9	0.03				
Plant type (13 days of water deprivation)	3	0.67	0.57	1.55	0.23	1.12	0.38
(CED-9) vs (161-1 Glurk G115)				2.5	0.04		

† Only significant orthogonal contrast interactions were reported (P<0.05); Letters in bold indicate significant differences at P<0.05

but not in CED–9 and 161–1 (0.5 & 0.8 μmol m^{-2} s^{-1}, respectively). On day 10, A in the 161–1 plants was significantly higher than the remaining types (Table I, Fig. 2).

Similar to A, plant transformation and drought stress impacted g_s rates. Transgenic plants maintained generally higher gs than the controls. Stomatal conductance declined significantly with the increase in drought stress and approached zero, 8 to 10 days after of water stress initiation (Fig. 3). Stomatal conductance did not differ among plant types under well watered conditions, however, CED–9 exhibited a significantly higher g_s on days 3 (14 to 45%) and 13 (80%) of water deprivation than the other types. Also, g_s were 50–80% higher in 161–1 on day 6 than the other types (Table I, Fig. 3).

Relative water content in transgenic and control plants responded in a similar manner to water deficit and was significantly impacted by drought (Table I, Fig. 4). RWC dropped from 89% in well–watered plants to 47% after 10 days of water deprivation.

Recovery from water stress. Recovery from drought stress was assessed every 2–3 days after re–watering, starting days 6 (plot 1), 8 (plot 2), 10 (plot 3) and 13 (plot 4). Analysis of variance (Table II) showed that while recovery of leaf RWC and g_s was more or less similar in all plant types, the recovery of A differed among plant types. Notable and

Table II. Analysis of variance and orthogonal contrasts results of photosynthesis (A), stomatal conductance (g_s) and relative water content (RWC) recovery from drought in controls (Glurk & G115) and transgenic (CED-9 and 161-1) tobacco plants after re-watering (days 8, 10, 13 and 17, for plots 1 through 4, respectively)

		A		g_s		RWC	
Source	df	F	P	F	P	F	P
Day	1	0.72	0.39	2.22	0.13	1.05	0.40
Plant type	3	6.40	0.00	1.25	0.29	0.62	0.69
Day* Plant type	3	4.30	0.00	2.72	0.04	1.90	0.46
Plant type (recovery of plot 4 at day 17)†	3	11.57	0.00	2.05	0.11	2.50	0.06
Glurk*day	3	13.0	0.00				
G115*day	3	15.4	0.00				
CED-9*day							
161-1*day							
Orthogonal contrasts (CED-9 161-1) vs (Glurk G115)	88	4.00	0.00				
G115 vs Glurk							
CED-9 vs 161-1	88	1.86	0.06				

†only significant orthogonal contrast interactions were presented in this table (P<0.05); Letters in bold indicate significant differences at P<0.05

significant differences were found among lines recovering after 13 days of water deprivation. CED–9 and 161–1 exhibited significantly higher values of A on day 17 than Glurk and G115. While CED–9 and 161–1 recovery was similar after 6, 8, 10 and 13 days of water deprivation, recovery of A in Glurk and G115 (Glurk*day & G115*day interactions in Table II) was significantly affected after prolonged period of water stress. Orthogonal contrasts (Table II) strengthened the results, indicating that trends in A in Glurk and G115 were similar and significantly differed from CED–9 and 161–1, the later did not differ among each other.

Percent recovery of leaves and the number of plants flowering after re–watering were assessed (Fig. 5). Whereas 84 and 82% of leaves in 161–1 and CED–9 recovered from drought, 70 and 67% in Glurk and G115 recovered, respectively. The most notable differences were observed in the number of plants flowering after re–watering, where 67% of 161–1 plants flowered compared to 33 and 30% of CED–9 and G115, respectively. Glurk had the lowest number of plants recovering, with only 17% of its individuals flowering after re–watering.

DISCUSSION

Gas exchange measurements provided significant physiological information on changes in CO_2 assimilation associated with plant transformation. Transformed tobacco plants did not differ significantly from controls before drought initiation. This is in general agreement with Dickman *et al.* (2001) and Awada *et al*. (2003) who reported undetectable morphological or physiological

Fig. 1. Average soil moisture content and standard error bars during water deprivation and re-watering (RW) on days 6, 8, 10 and 13 for plots 1 through 4 respectively

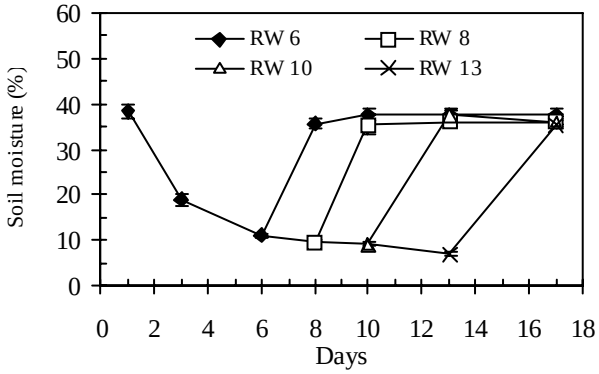
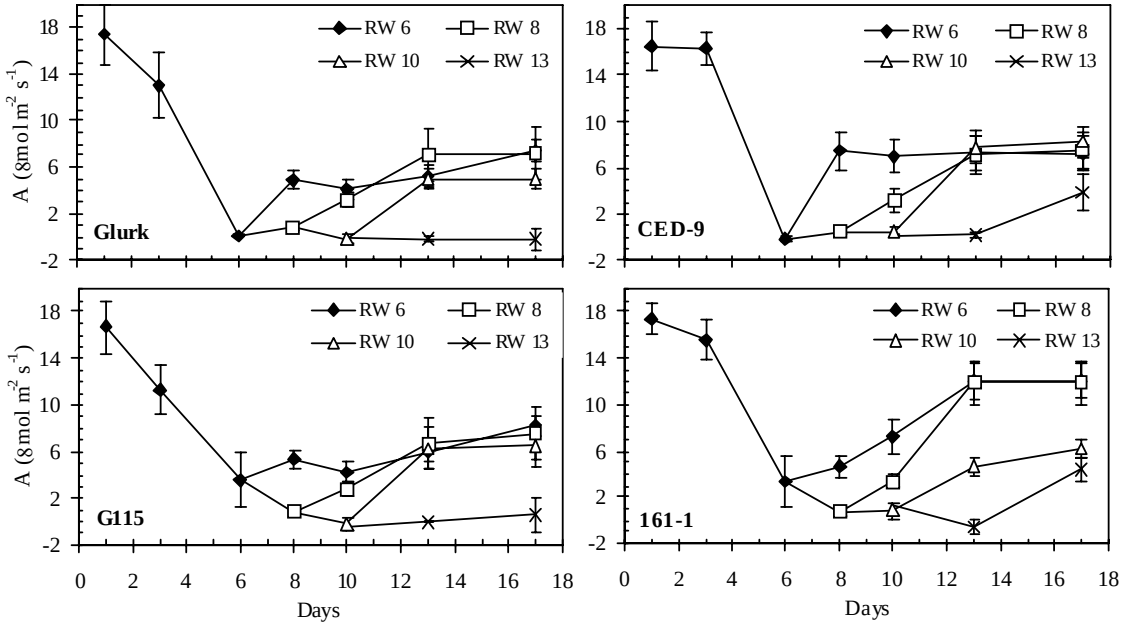


Fig. 2 Net photosynthetic rates (A μmol m^{-2} s^{-1}) and standard error bars during water deprivation and re-watering (RW) on days 6, 8, 10 and 13 for plots 1 through 4 respectively, in controls (Glurk and G115) and transgenic (CED-9 and 161-1) tobacco plants in the greenhouse.



changes in unstressed controls and lines expressing Bcl–xl, 161–1 or CED–9.

Drought is one of the major causes of osmotic stress in plants, disrupting plant functions on molecular, cellular and whole plant levels (Hasegawa *et al.*, 2000). Drought stress induced a significant and rapid decline in A and g_s three days after its initiation; this decline was steeper in controls than in transgenic plants. The slower decrease in A in transgenic plant can be associated with a more effective delay in dehydration (Chaves 1991; Volaire & Thomas, 1995). The decline in RWC lagged behind the A and g_s, observed on the 6th day of water deprivation. Similar findings were reported by Krampitz and Fock (1984) and Ferrario–Méry *et al.* (1998), suggesting that stomatal closure at early stages of drought stress leads to photosynthesis reduction, without affecting electron transport, mitochondrial activity and photorespiration. Ferrario–Méry *et al*. (1998) found that the decline in water contents of leaves lagged behind A and g_s and was observed 5 days after water deprivation in control and in tobacco lines expressing nitrate reductase. Recovery of plants after prolonged period of drought was enhanced by plant transformation. The fact that non–transformed Glurk and plants transformed with β–glucuronidase (G115) were similar and did not show any improvement in drought resistance, indicates that the enhancement in resistance was

Fig. 3 Stomatal conductance (g_s mol m^{-2} s^{-1}) and standard error bars during water deprivation and re-watering (RW) on days 6, 8, 10 and 13 for plots 1 through 4 respectively, in controls (Glurk and G115) and transgenic (CED-9 and 161-1) tobacco plants in the greenhouse.

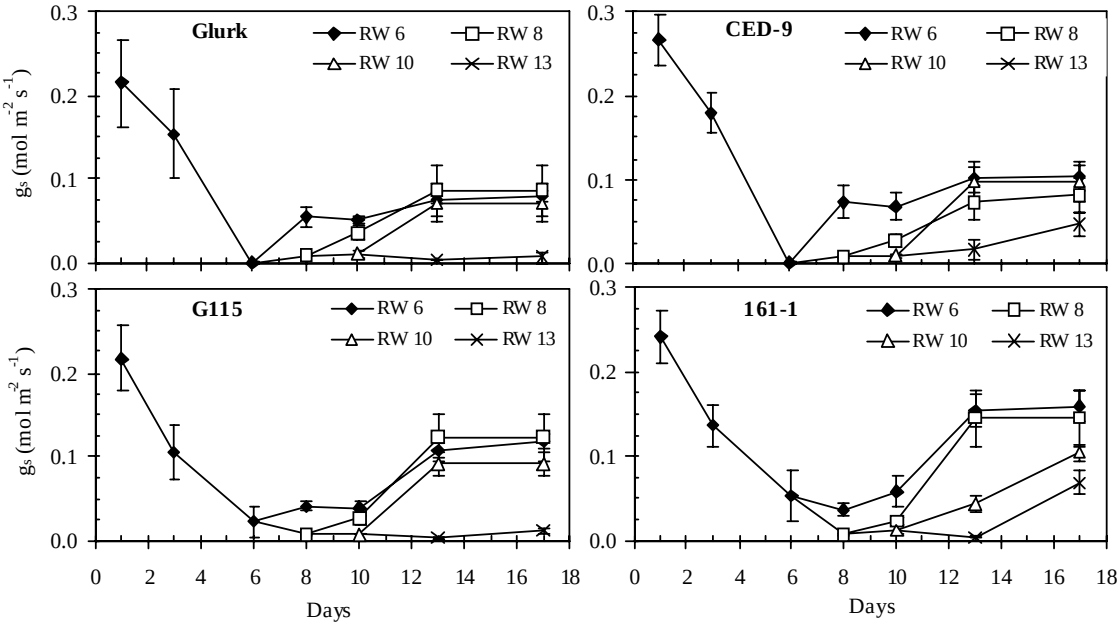
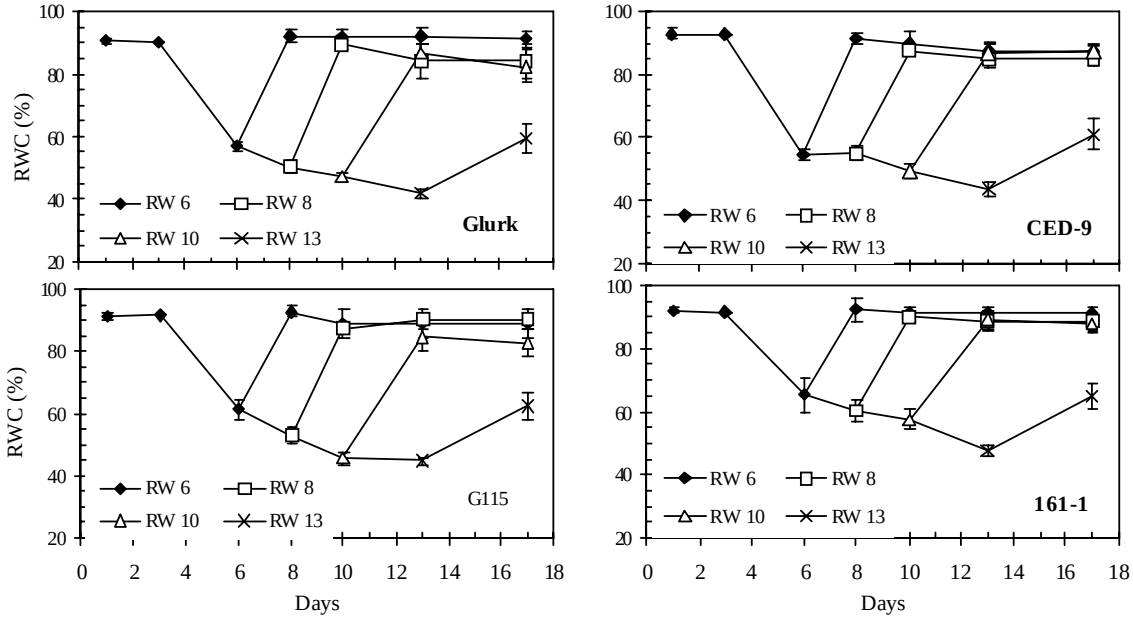


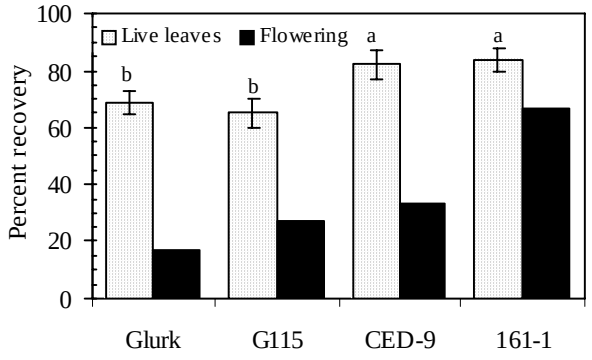
Fig. 4 Relative water content of the leaves (RWC %) and standard error bars during water deprivation and re-watering (RW) on days 6, 8, 10 and 13 for plots 1 through 4 respectively, in controls (Glurk and G115) and transgenic (CED-9 and 161-1) tobacco plants in the greenhouse.



specifically dependent on anti–apoptotic genes and not to the indirect consequence of transformation or presence of a foreign gene. Also, the differences observed between CED–9 and 161–1 in terms of resistance to drought may be related to the function of the introduced gene (Qiao *et al*., 2002)

It is unclear at this point as to what factors contribute to this enhancement in plant responses to drought stress in these lines. Drought stress is known to be associated with the release of ROS that cause degenerative reactions; disruption of membrane integrity and electrolyte leakage

Fig. 5 Recovery of leaves after 13 days of water deprivation with standard error bars, and percent of plants flowering after recovery from water deprivation in controls (Glurk and G115) and transgenic (CED-9 and 161-1) tobacco plants. Bars with the same letters are not statistically significant at P<0.05.



(McKersie *et al*., 1996). Severe drought stress was found also to induce protease enzymes that increase protein turnover (Ingram & Bartels, 1996). Tolerance to environmental stresses has been positively correlated with the levels of both antioxidant and the activities of oxygen free radical–scavenging enzymes (Bridger *et al*., 1994). Under stress and in the presence of anti–apoptotic genes, the maintenance of mitochondrial membrane integrity is essential for the generation of ATP (Qiao *et al*., 2002). This is crucial for the vacuole ATPase activity and necessary for photorespiration to prevent ROS accumulation in the chloroplasts. Such role of the anti–apoptotic genes prolongs the life of cells destined to die. Xiong and Zhu (2002) suggested improvement of drought tolerance in plants can be attained by the maintenance of homeostasis that is achieved by compounds like praline, detoxification of oxidative compounds (e.g. hydrogen peroxide & hydroxyl radicals) caused by ROS, and scavenging of ROS that reduce the damage on protein and DNA.

Interest in regulation of PCD has grown significantly with the establishment of their roles in development, and defense against pathogens and stress response in metazoans (Adams & Cory, 1998). Cell death suppressors have been shown to inhibit cell death induced by a broad range of stresses in tissue cultures of animals (Cotter & Al–Rubeai, 1995). In plants, these genes appear to enhance a broad spectrum of tolerances including, biotic stresses (Dickman *et al*., 2001), abiotic stresses (Li, Vaghichipawala, Dunigan & Dickman, unpublished data, Awada *et al*., 2003), radiation and herbicides (Mitsuhara *et al*., 1999), cold and salt (Qiao *et al*., 2002). In this study we have demonstrated the importance of anti–apoptotic genes for plant function in the presence of drought and recovery after re–watering.

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