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Response to water stress in transgenic (*p5cs* gene) wheat plants (*Triticum aestivum* L.)

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Abstract

Transgenic technology in plants has been used to introduce genes that perform as osmoprotection to obtain tolerant to abiotic stresses genotypes. Proline is considered as one of these osmoprotectors. Therefore, Δ1-pirrolyne-5-carboxylate synthesize (P5CS) enzyme, coded by *p5cs* gene is important and also a limiting factor in its pathway synthesis. This work aimed to evaluate proline accumulation in *p5cs* gene-transformed wheat plants. Experiment was conducted in randomized entirely design with 18 treatments (seven second generation (T_2) transgenic plants, nine first generation (T_1) transgenic plants and two checks with and without irrigation) and ten replications. Leaves were collected for conducting analyses of proline content, lipid peroxidation and water relative content every four days. The data were subjected to analyses of variance, using F-test at 1% and 5% probability levels and means grouped by Scott and Knott test at 5% probability level. The results showed that both transgenic and non-transgenic irrigated plants suffered reduction of water accumulation in relation to non-transgenic and plants without irrigation. However, they resisted eight days without water supply and with turgidity. Transgenic plants with constituted promoter (*Ubi*) and induced stress promoter (*aipc*) did not present significant differences in relation to proline levels. Lipid peroxidation values did not show any difference between transgenic plants and non-transgenic plants under no irrigation treatments during the evaluation period. The results indicated that transgenic plants produced approximately 1.85 more proline content than normal, demonstrating the *p5cs* gene expression.

Keywords: Abiotic stress, cereal crops, gene expression, proline, transformated plants.
Abbreviations: ROS_reactive oxygen species; MDA_malondialdehyde; Ubi_ constitutive promoter of corn Ubiquitine; aipc_induced stress promoter; WRC_water relative content; T_0_transformated plants; T_2_ 2nd generation of transformed plants.

Introduction

Wheat (*Triticum aestivum* L.) is a cereal that widely used in human and animal food around the world. Due to its plasticity for adaptation it can be cultivated in tropical subtropical and regions (Damalgo et al., 2009). In addition, wheat is an important crop in rotation and crop succession in agricultural production units, ensuring economic stream and farmer's sustainability. According to the United Nations Organization for Food and Agriculture (FAO, 2014), there was an increase in world estimate cereals production, around 2,542 million tons, 20 million more than that produced in 2013 agricultural year.

Abiotic stresses, such as soil salinity, water deficit and extreme temperatures are among the main factors that limit plants growth and yield, resulting in high losses to world economy (Su and Wu, 2004). Generally, water deficit modifies the growth and development of cultivated plants, under which the yield is affected differentially by water availability and efficiency by plants (Santos and Carlesso, 1998).

The environmental stress can trigger transcription of some genes that may be widely classified into two groups: those that offer protection and the ones that regulate signal transduction and gene expression associated with other processes (Shinozaki and Yamaguchi-Shinozaki, 2000; Seki et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 2005).

Plants resistance to drought is really complex and its expression depends on the action and interaction of different morphological characters (reduced leaf area, winding leaf, wax content, efficient root system and stability of production); physiological characters (transpiration reduction, high efficiency of water use, stomata closure and osmotic adjustment) and biochemists characters (accumulation of proline, polyamines, diamine, increase in nitrate reductase activity, besides increase in carbohydrate storage). However, the knowledge of genetic mechanisms that condition these characters is limited (Mitra, 2001; Fumis and Pedras, 2002).

Obtaining genotypes tolerant to abiotical stresses by classical breeding methods is difficult, mainly due to low heritability and high interaction between genotype × environments. In addition, selection processes usually occur in non-uniform conditions (Smith et al., 1990). Therefore, plants transformation has demonstrated its capabilities as an excellent option for developing wheat genotypes tolerant to

drought and more productive (Sahrawat et al., 2003; Jones, 2005).

Increasing tolerance to water deficit stress, by genetic transformation, has been obtained for several species using two main strategies: genes introduction involved in synthesis of osmoprotection molecules or by coding genes of transcription factors (Molinari, 2006). Several mechanisms controlling plant protection against drought, high temperatures and saline soils are known yet. One of them is proline accumulation that is a protein with protective function against the stresses hyper osmotic (Zhu et al., 1998; Hong et al., 2000; Su and Wu, 2004). Increasing the proline synthesis in plants may aid promote oxidative stress tolerance (Molinari, 2006).

Molecular genetics and genetic engineering has contributed to plants improvement (Pellegrineschi et al., 2002). The studies of important agronomic traits and their behavior in plants subjected to abiotic and biotic stresses allow the assessment of plant responses to these conditions. Such knowledge makes the transgene technology as a strategy obtain information about stress physiology (Vendruscolo, 2005). In wheat specie (*Triticum aestivum* L.), *p5cs* gene was introduced into genome through *Agrobacterium tumefaciens* and transgenic plants subjected to salt stress showed an increase in tolerance (Sawahel and Hassan, 2002). Vendruscolo (2005) obtained twelve lineages of national wheat transformed with *p5cs* gene. The gene induced tolerance to two types of abiotic stress: low temperature and water deficit.

Several studies have demonstrated that genetic manipulation for the higher gene expression and proline production (*p5cs*) increases the production of its osmolyte that acts during stress period, and consequently, results in higher tolerance to it (Kishor et al., 1995; Zhu et al., 1998; Molinari et al., 2004; Vendruscolo, 2005; Molinari, 2006).

Plants genetic transformation has designed to increase tolerance to different types of abiotic stresses based on introduction of osmoregulation genes and transcription factors of genes regulated by abiotic stresses (Shinozaki and Yamaguchi-Shinozaki, 2005). Some strategies use the introduction of one or several genes involved in signaling pathways and regulation or encoding enzymes, resulting in synthesis of compounds that protect cellular structures, such as osmolites and antioxidants (Shinozaki and Yamaguchi-Shinozaki, 2005).

Therefore, this work aimed to evaluate seven wheat lines (*Triticum aestivum* L.) in T_2 generation, as well as to demonstrate proline action mechanism, in relation to the osmotic adjustment and protection of cell membranes.

Results and Discussion

Genetic analysis of T_1 plants

The genetic segregation analyses of *p5cs* gene in T_1 generation were developed based on the expression of *bar* gene that confers herbicide resistance in all plants transformed by the plasmid, as shown in Table 2.

Observed segregation in S-6-16, S-84 and S-6-4 transformed families fit the ratio of 3:1, indicating the presence of a dominant gene (Table 3). This segregation ratio was not significant at 5% probability level, a fact that suggests the insertion of a single copy. However, S-6-1 transformed plants, presented 1:1 segregation ratio, non-significant at 5% probability level, indicating a differentiated pattern of transgene expression. All transformed plants showed proline expression, demonstrating that the presence of one more copy of gene did not result the silencing effect of transgene in T_2 plants.

Evaluation to water stress tolerance

T_2 transgenic plants and control, subjected to water stress, differed significantly (1% probability level) for proline content (Table 4). Proline levels in transgenic plants and control without water stress (0 day) were similar and ranged from 1.38 μmoles g^{-1} of dry matter in transgenic plants to 1.49 μmoles g^{-1}f dry matter in control plants (Fig 1).

Maximum levels of proline were presented by pUbi S2-34 and S-6-1 genotypes, when subjected to eight days of water stress, which accumulated 5.37 and 5.25 μmoles g^{-1} of dry mass, respectively. The results evidenced a maximum increase of 1.85 times in proline content between transgenic plants and control. Several authors have described proline increase in plants of distinct species, transformed with *p5cs* gene when submitted to water stress, including wheat (Molinari et al., 2004; Vendruscolo, 2005; Molinari, 2006).

Genotypes pUbi although possessing constitutive promoter and continually express *p5cs* gene, did not show high levels of proline in water stress absence. Thus, such observation could be clarified as a result of pUbi also constitutes an induced stress promoter. Christensen and Quail (1996) concluded that mechanical and thermal stresses caused a significant increase in *gus* gene expression in rice when it used maize ubiquitin promoter.

Water process stress provides stomata closing, reducing the supply of CO_2 to chloroplasts and promoting the reduction of photosynthetic activity, a fact that results to the formation of reactive oxygen species (ROS). Consequently, the lipid peroxidation may result in rupture of cell membranes in plants subjected to stress (Hernandez et al., 2000; Mundree et al., 2002; Parvanova et al., 2004). Quantification of lipid peroxidation through indirect methods, such as content of malondialdehyde, is used as a measuring criterion of stress at the cellular level (Parvanova et al., 2004). This experiment showed that transgenic plants had higher MDA content, suggesting a reduced oxidative damage protection.

Regarding to MDA (malondialdehyde) content, the transgenic and control plants subjected to stress, differed significantly (F-test) during the stress only at 0 and 2 times similar to other studied periods (Table 5). During the absence of water stress, MDA values ranged from 0.05 mmoles g^{-1} of fresh mass in transgenic plants and 0.06 mmoles g^{-1} of fresh mass in control plants. At 8th day without water supply, the values were 0.07 mmoles g^{-1} of fresh mass in control plants. Although values are similar, plants subjected to water stress showed higher levels of MDA with difference statistic in comparison to irrigated control plants (Fig 2). The eighth day of water stress showed 0.062 mmmoles g^{-1} of fresh mass for S-6-4 plants as the most inferior MDA value obtained for transgenic plants, whereas the highest value was 0.093

Table 1. Transformed plants (T_2) and its respective families.

Transformed plants	Families
S-84	(134, 101, 48, 18, 15)
S-88	(136, 124, 90, 72, 61)
S-6-4	(5, 4, 3, 2, 1)
pUbi S2	(34, 32, 30, 26, 13, 12, 11, 10, 8, 6)
S-6-14	(109, 105, 78, 53,24)
S-6-16	(135, 91, 75, 54, 9)
S-6-1	(3, 2, 1)

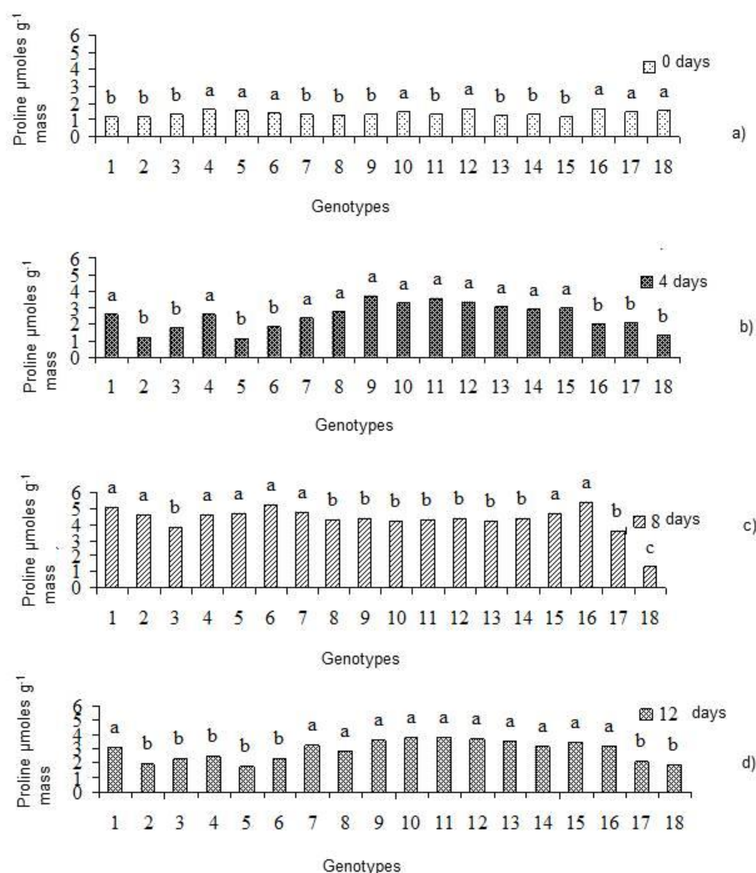

Fig 1. Proline content (µmoles g^{-1} of dry matter) in plants subjected to water stress at 0 (a), 4 (b) and 8 (c) days and after irrigation (d). Events from 1 to 16 = transformed plants, (1 to 6: promoter water stress; 7 to 16: constitutive promoter). Event 17 = control under water stress. Event 18 = irrigated control. Means followed by the same letter do not differ significantly by Scott Knott test, at 5% probability level.

mmmoles g^{-1} of fresh mass for S-6-14 plants. On the other hand, control plants submitted to stress showed MDA value around 0.069 mmmoles g^{-1} of fresh mass. The control plants presented 0.052 mmmoles g^{-1} of fresh mass when irrigated, demonstrating a greater integrity and maintenance of physiological processes, an expected result since these ones have not suffered stress. This experiment evidenced that transgenic plants presented higher MDA content, suggesting a reducing protection to oxidative damage. These results disagreed from other studies that showed highest proline content and inferior MDA content by an imposition of water restriction (Vendruscolo, 2005).

This fact indicates that despite of high content in proline accumulation among transgenic plants, there was no efficient mechanism for cell membrane protection against oxidative stress.

Proline content in transgenic plants with constitutive promoter (Ubi) and induced stress promoter (*aipc*) did not present any significant difference. Results indicated that MDA values did not differ between transgenic and no transgenic plants under water stress during evaluation period.

Analysis of water relative content (WRC) revealed significant differences by F test at 1% probability level among control plants and events (Table 6). Considering these results, it is observed that likewise the transgenic plants, control ones could resist eight days without water supply, showing turgidity. In the absence of water stress, transgenic and control plants showed values ranging from 85.02 % to 89.39 %, respectively, with no significant difference (Fig 3). Throughout the water stress process, in the period of four and eight days, both transgenic and control plants, subjected

Table 2. Identification of evaluated treatments.

Identification of evaluated treatments	Transformed plants
1	S-88
2	S-16
3	S-84
4	S-6-14
5	S-6-4
6	S-6-1
7	pUbi S2-6
8	pUbi S2-8
9	pUbi S2-10
10	pUbi S2-11
11	pUbi S2-12
12	pUbi S2-13
13	pUbi S2-26
14	pUbi S2-30
15	pUbi S2-32
16	pUbi S2-34
17	Control, without irrigation
18	Control, with irrigation

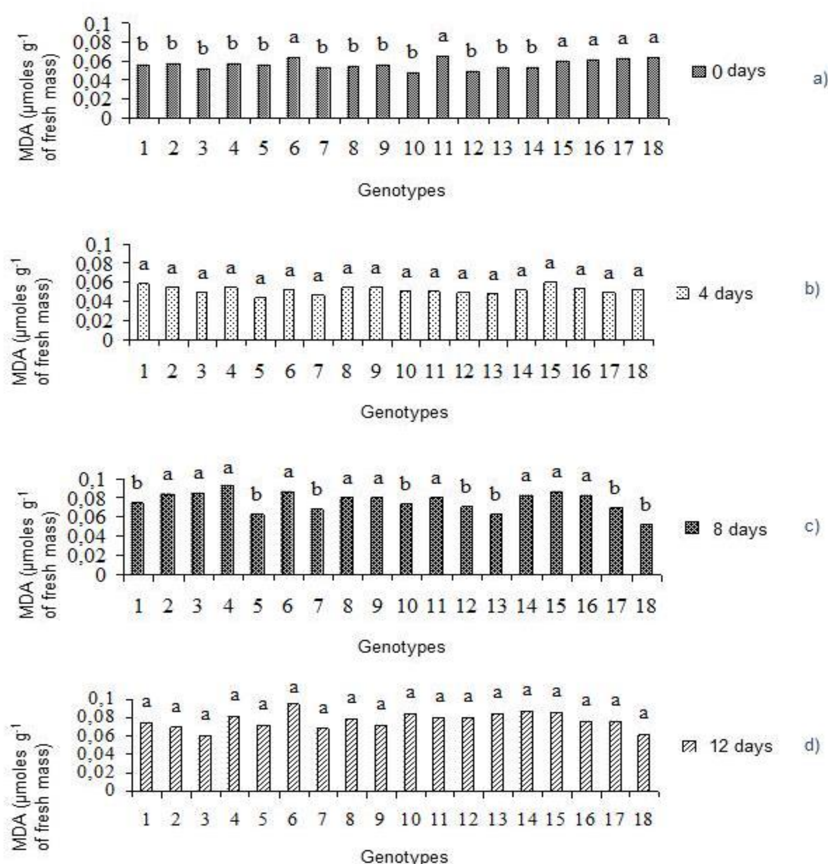

Fig 2. Levels of membrane peroxidation (MDA in mmoles g^{-1} of fresh mass) obtained in plants subjected to water stress at 0, 4, 8 days and after irrigation. Events from 1 to 16 = transformed plants (1 to 6: induced stress promoter; 7 to 16: constitutive promoter). Event 17 = control under water stress. Event 18 = irrigated control. Means followed by the same letter do not differ significantly by Scott Knott test at 5% probability level.

to water stress demonstrated a reduction in water relative content (WRC). Transgenic plants showed values that ranged from 37.14% (pUbi S2-32) to 65.24% (S-6-4). On the other hand, control stressed plants showed 57.47% of WRC, whereas control irrigated plants presented 84.74% (Fig 3).

Materials and Methods

Plant material and cultivation conditions

Transgenic wheat plants containing *p5cs* gene used in this experiment were obtained from Universidade Federal do Paraná, Brasil.

Table 3. Segregation ratio of events obtained by transformation with *p5cs* gene in T_1 generation with induced stress promoter.

	Progenies Analysis (T_1)		Segregation Ratio	χ^2	Probabilility (%)
	Resistant	Susceptible			
S-6-1	3	5	1:1	0.50	47.95
S-6-16	140	59	3:1	2.29	12.99
S-84	134	35	3:1	1.65	19.77
S-6-4	51	15	3:1	0.18	66.98

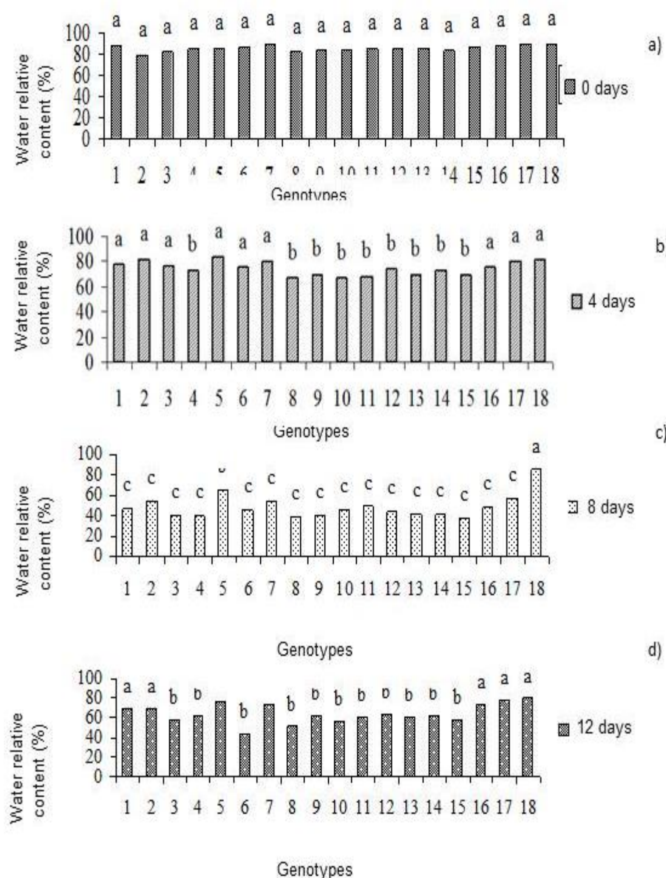

Fig 3. Levels of water relative content (%) obtained in plants submitted to water stress at 0 (a), 4 (b) and 8 (c) days and after irrigation (d). Events from 1 to 16 = transformed plants (1 to 6: induced stress promoter; 7 to 16: constitutive promoter). Event 17 = control under water stress. Event 18 = irrigated control. Means followed by the same letter do not differ significantly by Scott Knott test at 5% probability level.

In 2005 agricultural year, 12 transformed plants (T_0) with *p5cs* gene tolerant to water stress were obtained and lately multiplied. The present study evaluated seven of these plants transformed in T_2 (2nd generation of transformed plants), as shown in Table 1. The experiments were performed at Laboratory of Biotechnology of Cooperativa Central de Pesquisa Agrícola in 2006 year. The pUbiS2 plants were obtained by co-transformation between pUbi*p5csf129a* plasmids (Zhang et al., 1995), presenting code sequence of *p5cs* gene positioned below the constitutive promoter of maize Ubiquity (*Ubi*). *Vigna aconitifolia* cDNA, showing replacement of phenylalanine by alanine at 129 position and, with TNOS sequence (nopaline synthase terminator sequence of *Agrobacterium tumefasciens*, and p*AHC20* plasmid containing bar gene), was used as a check mark under the control of constitutive promoter of maize Ubiquity (*Ubi*) (Christensen and Quail, 1996). Remaining plants were transformed with pJS107 plasmid, containing the compound transgene by promoter stress induced *aipc* and code sequence *p5cs* gene from *Vigna aconitifolia* L. (Hu et al., 1992). The *aipc* promoter is ABA induced, possessing an ABA element responsive with 49 bp from *hva 22* barley gene (Shen and Ho, 1995), minimum promoter of acting *act1* with 100 bp (Su et al., 1998) and an intron of *hva 22* (Shen and Ho, 1995). The *bar* gene of glifosinato ammonium tolerance was used in transgene as a selection marker under the promoter control CaMV 35 (Hu et al., 1992).

Evaluation of T_1 plants resistant to Glufosinate ammonium

T_1 genotypes were multiplied and their progenies were evaluated for resistance to ammonium Glufosinate herbicide. Seeds were sown in a tray with sterile soil under greenhouse conditions. After 15 days of initial seed germination, seedlings were sprayed with aqueous solution 1% (v/v) of the same herbicide. After a week, the evaluation of seedlings susceptibility and resistance was conducted. The seedlings with healthy growth and green leaves, even with herbicide application considered resistant. Data were analyzed using

Table 4. Values and significance of medium squares (MS) in T_2 wheat transgenic plants and control based on proline content, subjected to 0, 4, and 8 days of water stress and after irrigation initiation at 12nd day.

Variation Sources	Periods of induced water stress						After irrigation	
	0 day		4th day		8th day		12nd day	
	FD	MS	FD	MS	FD	MS	FD	MS
Replications	9	0.04	9	0.69	9	0.82	9	0.97
Genotypes	17	0.26**	17	5.84**	17	4.92**	17	3.07**
Error	144	0.06	142	1.11	124	0.47	89	0.66
Total	170		168		150			115
Mean	-	1.39	-	2.46	-	4.24	-	2.59

VS = Variation Sources; FD = Freedom degrees; MS = Medium Square; ** significant at 1% probability level, by F-test.

Table 5. Values and significance of medium squares (MS) for lipid peroxidation (MDA) in T_2 wheat transgenic plants and control, subjected to 0, 4, and 8 days of water stress and after irrigation initiation at 12nd day.

Variation Sources	Periods of induced water stress						After irrigation	
	0 day		4th day		8th day		12nd day	
	FD	MS	FD	MS	FD	MS	FD	MS
Blocks	9	0.000007	9	0.00009	9	0.00026	9	0.0021
Genotypes	17	0.00027**	17	0.00015ns	17	0.00088**	17	0.0073ns
Error	149	0.00007	149	0.00012	148	0.0003	237	0.0005
Total	175		175		174		254	
Mean	-	0.056	-	0.051	-	0.077	-	0.078

VS = Variation Sources; FD = Freedom degrees; MS = Medium Square; ** significant at 1% probability level, by F test; ns = no significant.

Table 6. Values and significance of medium squares for water relative content (WRC) in T_2 wheat transgenic plants and control, submitted to 0, 4 and 8 days of water stress and after the beginning of irrigations at 12nd day.

Variation Sources	Periods of induced water stress						After irrigation	
	0 day		4th day		8th day		12nd day	
	FD	MS	FD	MS	FD	MS	FD	MS
Blocks	9	53.97	9	133.42	9	257.28	9	111.72
Genotypes	17	68.02ns	17	280.15*	17	1039.75**	17	687.28**
Error	145	48.27	149	135.91	149	201.66	112	218.07
Total	171		175		175			138
Mean	-	85.33	-	74.57	-	47.71	-	64.24

VS = Variation Sources; FD = Freedom degrees; MS = Medium Square; ** significant at 1% probability level, by F test; * significant at 5% probability level, by F test; ns = no significant

Chi-square (χ^2), to calculate ratio probability according to expected by Mendelian segregation patterns, at 5% probability level.

Obtaining T_2 plants

T_2 seeds were sown in pots containing substrate (50% soil: 30% organic fertilizer: 20% sand), maintained under greenhouse conditions with temperatures ranging from 25°C to 26°C. Experiment was conducted in randomized entirely design with 18 treatments, each one possessing 12 pots with three plants per pot.

After 15 days of initial seed germination, herbicide application (solution of 25 mL-1) was performed for selecting transformed seedlings with Glufosinate ammonium tolerance. The pUbi S2 plants, since were obtained by co-transformation, did not receive selective test application and; therefore, all plants were considered in the evaluation. Thinning process was conducted in treatments that showed all resistant plants to herbicides, resting only one plant per pot.

Among the events, in which not all families were tolerant to herbicide or showed heterozygosity, 25 plants were selected for each event. Under the same growing conditions, 24 control plants, genetically untransformed, belonging to CD 200126 lines were sown. From 24 plants control, 17 plants were subjected to water stress and the other ones were irrigated during the same period. Table 2 presents evaluated transgenic plants.

Water stress application

Plants were subjected to water deficit when reached booting phase, approximately 58-65 days after germination (Zadocks et al., 1974). Control and transformed plants were subjected to a period of eight days with water stress, returning to irrigation at ninth day. Pots were arranged at greenhouse, in similar positions in relation to solar radiation incidence. Leaves collected in order to conducted analyses of relative water content (RWC), proline and MDA (Malondialdehyde) were developed every four days.

Physiological characteristics evaluated

Physiological analyses were performed at Biotechnology Laboratory belonging to Coodetec in Cascavel County, Paraná State. Leaves collection was standardized according to the methodology proposed by Vendruscolo (2005). To conduct proline analysis the flag leaves were always collected. For MDA evaluation the second leaf below the flag leaf was used. For relative water content (RWC) the third leaf below the flag leaf was analyzed. Collections were conducted during morning period at early hours of the day. Material used for proline and MDA analysis after sampling, was immediately weighted and stored at a temperature of -80°C. Leaves for analysis of relative water content were weighted and immediately dried.

Relative water content (RWC)

Evaluations about relative water content (RWC) were conducted according to the methodology proposed by Saíram et al. (1998), using the third fully expanded leaf of each plant. After collecting, leaves were stored in plastic bags and sent to the laboratory for determining leaves fresh mass.

Turgid mass was obtained after leaves imbibition for 16-18 hours in distilled water under environmental temperature and in glass packaging previously washed and autoclaved. After this period, leaves were quickly dried with paper towels and weighted.

Dry mass was obtained after material drying in forced air chamber at 70°C for 72 hours. The WRC was calculated according to Schonfeld et al. (1988) equation, as follow:

$$WRC\ (\%) = (FM - DM) / (TM - DM) \times 100$$

Where;
FM = Fresh Mass
DM= Dry Mass
TM = Turgid Mass

The water relative content (WRC) was determined to evaluate the maximum period of turgidity maintenance of cells in transformed and untransformed plants.

Lipids peroxidation level

Lipids peroxidation level was measured in terms of malondialdehyde contents (MDA) using thiobarbituric acid reaction (Saíram et al., 1998). Therefore, for this analysis the second leave of each plant was used, weighing about 0.25 g of leaf tissue that was stored in aluminum foil at a temperature of -80° C during collections periods.

In order to determine MDA content, samples were homogenized in 5 mL of 0.1% trichloroacetic acid (w/v) and centrifuged at 14,000 rpm for 5 minutes at environment temperature. Subsequently, 2 mL of supernatant were removed from each sample, 4 mL of thiobarbituric acid solution (0.5% in 20% (w/v) of trichloroacetic acid) was added. Then, this mix was maintained in bain-marie at 100° C for 30 minutes. Samples were centrifuged again for 5 minutes at 14,000 rpm for particles sedimentation of plant tissue (Saíram et al., 1998).

Absorbances at 532 and 600nm wavelengths of each sample were read in spectrophotometer. Reading at 600 nm was used for not specific turgidity correction. The MDA content was calculated by the formula shown below, where the 155 $mmol^{-1}\ cm^{-1}$ values corresponds to extinction coefficient.

$$MDA = (A_{532} - A_{600}) / 0.155$$

Proline quantification

Proline quantification was performed using the flag leaf of each plant (Bates et al., 1973). Aapproximately, 0.025g of leaf material was weighed for proline analysis in two repetitions. Another sample of the same mass was weighted to determine dry mass. Later, samples were packed in aluminum foil and stored in freezer at -80° C, during collection period. Proline levels in plants tissues were determined by methodology proposed for Bates et al. (1973), with modifications. A 0.025 g of plant tissue was macerated in 5 mL of 10% sulfosalicylic acid solution. Solution was transferred to falcon tubes of 15 mL and centrifuged at 7500 rpm for 5 minutes. Then, 2 mL of supernatant was transferred to new tubes and 2 mL of ninhidrin acid and 2 mL of glacial acetic acid were added. Tubes were kept in bain-marie at 100° C for 60 minutes, with final reaction conducted on ice bath. After thermal shock, 4 mL of toluene was added and solution was homogenized for about 20 seconds in Vortex for complete proline extraction. Absorbance at 520 nm, of each sample of its material, was determined on spectrophotometer, using toluene as white. Proline concentration was determined from a standard curve and calculated on the basis of samples dry weight. Materials designed for obtaining dry mass were placed in forced air chamber at 70° C and dry mass value obtained after 24 hours (Bates et al., 1973).

Statistical analysis

The analysis of variance was performed using F test at 1% and 5% probability level. Subsequently, means of treatments were compared by Scott and Knott test (1974) at 5% probability level. The analyses were developed using Genes computer program (Cruz, 2013).

Conclusion

Transgenic plants produced approximately 1.85 times more performance than non-transgenic plants, when subjected to water stress, demonstrating the expression of *p5cs* gene. Proline content in transgenic plants with constitutive promoter (Ubi) and stress induced promoter (aipc) did not show significant differences and MDA values did not differ between transgenic plants and no transgenic plants with water stress, during the evaluation period.

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