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Molecular and physiological responses to water deficit in wheat (*Triticum aestivum* L.)

Respuestas fisiológicas y moleculares al déficit hídrico en trigo (Triticum aestivum L.)

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Abstract

Physiological and molecular responses of five wheat genotypes: one canadian cultivar, with low temperature tolerance, and 4 experimental lines, which demonstrated in the field high yield under drought stress were studied under water stress. We determined leaf relative water content and gene expression of rob 5 and dehydrins in response to low water availability. One genotype showed a higher RWC with less leaf fresh weight and water soil content. There were not differences between genotypes in expression genes but, we found differences in gene expression between both tested genes. Rob 5 expressed in control as well as in stressed plants, while dehydrins only expressed in stressed plants and its expression increased as the water content of leaves decreased.

Key words: water stress , Rob 5, dehydrin, *Triticum aestivum* L.

Resumen

Las respuestas fisiológicas y moleculares de cinco genotipos de trigo: un cultivar canadiense, tolerante a las bajas temperaturas y 4 líneas experimentales argentinas, que habían mostrado, un alto rendimiento a campo bajo condiciones de deficiencia hídrica, fueron estudiadas en condiciones de baja disponibilidad de agua. Se determinó el contenido relativo de agua en las hojas y la expresión de 2 genes, rob 5 y dehidrinas, en respuesta a la baja disponibilidad de agua. Uno de los genotipos mostró mayor CRA con un menor contenido de agua en las hojas o en el suelo. No hubo diferencias genotípicas en la expresión de los genes sin embargo, fue diferente la expresión de éstos, en respuesta al estrés hídrico, dado que el gen Rob 5 se expresó tanto en las plantas control como en las estresadas, mientras que el de las dehydrinas sólo se expresó en las plantas bajo estrés, y su expresión aumentó en función de la disminución del contenido relativo de agua de las hojas.

Palabras clave: sequía, Rob 5, dehidrinas, *Triticum aestivum* L.

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Introduction

Drought is a major factor limiting crop productivity world-wide and cultivating plants with increased resistance to this stress appears critical to keep yields at a sufficient level. The screening of such cultivars based on their productivity, is a long and tedious task that would undoubtedly be accelerated if traits that could be reliably related to water were identified.

Whole plants respond to drought through morphological, physiological, and metabolic modifications occurring in all plant organs. At the cellular level plant responses to water deficit may result in cell damage, whereas other responses may correspond to adaptative processes. Although a large number of drought-induced genes have been identified in a wide range of plant species, a molecular basis for plant tolerance to water stress remains far from being completely understood (Ingram and Bartels, 1996).

Water deficit is intrinsic to most abiotic forms of stress- not only during drought, but also at low temperature and when the soil contains high concentrations of ions. Changes in gene expression are involved in physiological responses to water stress, but the method by which specific genes might play a part in stress is unknown. Such lack of progress is mainly due to the multigenic nature of sensitive and tolerant phenotypes. In addition, different plant species have a variety of mechanisms that have evolved in a family-specific or order specific manner to confer tolerance. The stress response can also vary depending on the developmental stage during which a plant is subject to stress.

Breeding programs to improve salt and drought tolerance are particularly important in countries that would benefit most from stress-tolerant crops. Conventional breeding approaches alone will be valuable, but it would be more beneficial to include as molecular markers the genes for the mechanisms associated with stress tolerance.

Among the water-stress induced proteins so far identified, dehydrins, the D-11 subgroup of late-embryogenesis-abundant (LEA) proteins (Dure *et al.* 1989; Wood and Goldsbrough, 1997) are frequently observed, and more than 65 plant dehydrin sequences are available (Close, 1997). Dehydrins are a group of proteins that accumulate during cold acclimation (Robertson *et al.* 1994 b; Danyluk *et al.* 1994), water stress (Close *et al.* 1989) and heat tolerance (Robertson *et al.* 1994 a). Dehydrins are heat stable, highly hydrophylic proteins that are thought to help stabilize other proteins and membranes during periods of cellular dehydration (Close *et al.* 1993a). These proteins display particular structural features such as the highly conserved Lys-rich domain predicted to be involved in hydrophobic interaction leading to macromolecule stabilization. Very little is known about dehydrin functions in plants (Cellier *et al.* 1998). Studies have established correlation between drought adaptation and dehydrin accumulation in wheat and poplar (Labhilili *et al.* 1995; Pelah *et al.* 1997) and sunflower (Cellier *et al.* 1998). Rob 5 is a gene that was studied in wheat in response to cold stress, it was observed that its expression increase in response to low temperatures (personal communication).

In most of the published studies gene expression was described as a function of time after the stress was applied rather than as a function of parameters describing the plant water status.

Therefore, it is difficult to determine from these data precise relationship between plant physiological responses to drought-induced gene expression (Cellier *et al.* 1998)

The present work was conducted in order to determine plant physiological responses and the expression of rob5 and dehydrin gene in four Argentinean experimental wheat lines that showed a good yield growth in a field under water stress and a Canadian cultivar that had a good performance under low temperatures.

Materials and Methods

Seedling growth conditions

Seeds of four Argentinean experimental lines supplied by Ing. Agr. Rubén Miranda (Dpto. of Agronomy, UNSur, Argentina): 425-94 (line 1), 873-97 (line 2), 890-97 (line 3); 898-97 (line 4) and a Canadian cultivar, AC domain, (cultivar 5) were sown on 18/1/2002, and seedlings were grown in a greenhouse under 18 h of light, 20/23°C (night/day) and the pots were filled with composite soil (terralite: soil, 1:3). We carried out two kind of experiments with the object to generate a different type of stress: Experiment I, leaves of well watered plants were dehydrated on the bench. We took samples to determine fresh weight, relative water content (RWC) and part of the material was frozen for immunoblot analysis at different times, at the start of the experiment, two and five hours later, 20 days old seedlings were used in the experiment.

Experiment II: On 25 February we watered all the pots, then, we separated those into two groups referred to as control watered during all the experiment and stressed plants were subject to progressive drought by withholding water. Control and stressed leaves were harvested after 10, 14 and 20 days of stress, first, second and third sampling. Then the rest of stressed plants were rewatered, and 72 hours later were harvested, fourth sampling time. At each sampling time were collected all the leaves from individual plants of each line, for physiological measurements and frozen separately for subsequent inmunoblot analysis with rob 5 and dehydrin gene.

Leaf Relative water content

RWC were estimated using the following formula: RWC= (FW - DW) / TW - DW) x 100, where FW= weight of freshly collected material, TW = weight after rehydration for 24 hours at 4° C in the dark and DW = weight after drying at 60 ° C for 24 hours.

Soil water content

Each pot was weighed daily at 10 AM and gravimetric soil water content was measured as grams of water per gram of oven - dried soil.

Protein isolation, SDS-PAGE electrophoresis and Western Blotting

Leaves of control and stressed plants after the harvest were rapidly frozen in liquid N_2. Then, they were homogenated, 500 mg fresh weight in 1.5 ml of buffer of Sodium borate, pH 8.0, containing sodium borate 0.95 gr, L-ascorbic acid 0.088 gr, 0.5 M EDTA 100 ml, b mercaptoethanol 500 ml, e - amino - caproic acid 0,333 gr, 20 % SDS 2.5 ml in a final volume of 50 ml. Then, leaves were disrupted in a motorized ground homogenizer. The 13000 rpm (90 min) supernatant fraction was collected for protein assay, SDS-PAGE electrophoresis and immunoblot analysis.

Electrophoresis on denaturing polyacrylamide gels was performed by the method of Laemmli (1970) with a 4% (w/v) stacking gel and a 12.5 % (w/v) resolving gel. Equal mass of protein (60 mg) were loaded on the gel. We did two equal gels, one to stain with coomasie blue and the other for western blotting. For immunological detection, proteins were fixed and elctrotransferred to nitrocellulose in a buffer containing 25 mM Gly at a constant current of 90 volts. Blots were blocked with 2% dry milk in Tris-buffered saline, incubated with rabbit anti - peptide antibodies followed by a goat anti-rabbit IgG alkaline phosphatase conjugate. The primary antibody was antyrob 5 (sumninistrated by Plant and stres laboratory, University of Saskatchewan), or against a synthetic peptide (EKKGIMDKIKELPG) that is highly conserved in the C- termini of group 2 LEA/ dehydrin proteins (Courtesy of T. J. Close). The secondary antibody was detected using 4-nitroblue-tetrazolium chloride and 5-bromo 4-chloro 3-indolyl-phosphate.

Results and Discussion

Physiological Characterization of drought stress

Experiment I

When leaves of well watered seedlings were dehydrated on the bench, apparently line 2 showed a lower lost of water in function of dehydration time, then it had the highest RWC after five hours of dehydration (Figure 1) and interestedly, when we related RWC in function of leaf fresh weight we found that apparently genotype 2 maintained a higher RWC with lower fresh weight (% control) (Figure 2). Water loss of excised leaves is a technique that was suggested for drought screening (Clarke and McCaig, 1982). Winter *et al.* (1988) showed in wheat, that water loss of excised leaves was correlated with an index of drought susceptibility (S), (least loss = lowest S), then we can say that under these conditions, genotype 2 is more tolerant to drought conditions.

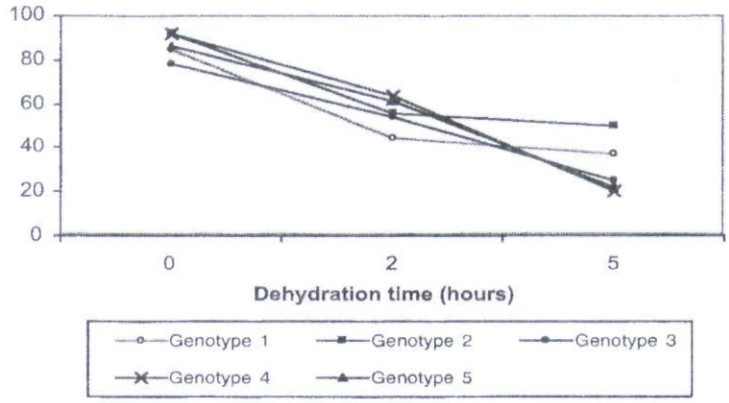

Figure 1. Leaf RWC (%) in function of dhydration time. Determinations in 20 days old seedlings, were made at the start of the experiment (time 0), two (time1) and five (time 2) hours later.

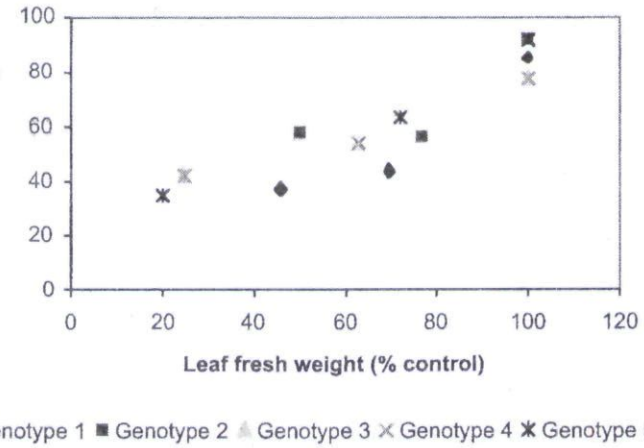

Figure 2. Variation of RWC (%) in function of leaves fresh weight (% control). Leaves of well watered plants were dehydrated on the bench during five hours. Values are the means from four repetitions.

Experiment II

During the development of the water stress, soil water loss increased progressively and differently in pots of the different genotypes. At the end of the experiment line 2 showed the lowest value of soil water content ($P < 0,05$) (Table 1).

Leaf relative water content was different between lines after withholding water. Changes in RWC during water stress development are shown in Table 2. At the time water was withheld (day 0) the five genotypes displayed similar values, but, at the end of the experiment the relative water content was 79, 54, 66, 62 and 67% for lines 1, 2, 3, 4 and 5 respectively. Then line one had highest leaf RWC under water stress while line 2 the lowest value ($P < 0.05$) (Table 2) Line 1 lost less water from the soil as well as from its leaves during water stress, while lines 2 lost the highest amount of water from the soil and reached the lowest RWC.

Table 1. Soil water content percent (%) at 10 (first sampling date), 14 (second sam pling date) and 20 (third sampling date) days after withholding water, in five wheat lines

	(%) Soil water content			
	Control	Stressed plants		
Genotypes		First sampling date	Second sampling date	Third sampling date
Genotype 1	43	40	30	16
Genotype 2	51	36	14	8
Genotype 3	54	40	23	11
Genotype 4	49	43	22	9
Genotype 5	52	38	25	10

Table 2. RWC (%) in leaves at 10 (first sampling date), 14 (second sampling date) and 20 (third sampling date) days after withholding water, in five gentypes of wheat

(%) Relative water content			
		Stressed plants	
Genotypes	First sampling date	Second sampling date	Third sampling date
Genotype 1	91	86	79
Genotype 2	93	83	54
Genotype 3	95	88	66
Genotype 4	96	88	62
Genotype 5	93	80	67

However, while the soil moisture contents after 14 days of drought were notably different (30 and 14 % for lines 1 and 2 respectively), at that sampling time the difference of leaf RWC between lines was minimal (86 and 83 % for lines 1 and 2 respectively); then, when we associated RWC of leaf in function of gravimetric soil water content, genotype 2 showed a highest RWC at a specific gravimetric soil water content (Figure 3). Therefore genotype 2 had the highest relative leaf water content in both types of experiments at the lowest leaf and soil water contents. The RWC differences according to genotype in wheat cultivars under water stress were also observed by Schonfeld *et al.* 1988; and Siddique *et al.* 2000, and the former authors suggest the use of RWC as a selection criterion for drought resistance in wheat (Schonfeld *et al.* 1988).

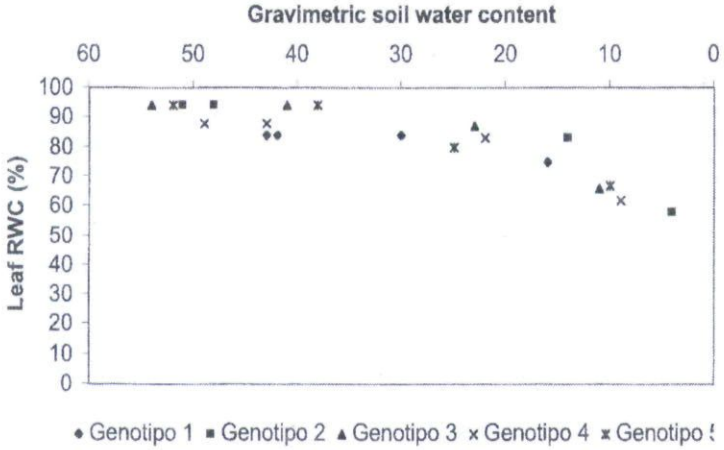

Figure 3. Soil and leaf water status of five genotypes plants during progressive drought initiated by withholding water. Leaf relative water content as a function of gravimetric soil water content (grams of water per gram of dry soil). Leaf relative water content and gravimetric soil water content values are the means from four repetitions.

Molecular Characterization of drought stress

The protein patterns obtained from stressed leaves were not obviously different from those of control leaves (results are not shown). The occurrence of rob5 and dehydrin antibody was followed in leaves of control and stressed plants in the different sampling dates. In all genotypes, the inmunoblots rob 5 (Figure 4 and 5) showed the presence of bands in control and stressed leaves, and did not increase its expression under water stress. The Rob5 immunoblot showed a band of about 42 - 45 kD polypeptides. This gene was present in the five genotypes in control and stressed leaves, having similar response in all the genotypes in different drought times. An increase in rob 5 expression gene was found under cold treatment in wheat (personal communication, Russell Trischuk). These proteins were observed in *Bromus inermis* as the most abundant set of heat - stable polypeptides in the cell fraction isolated from ABA - treated cells (Robertson, *et al.* 1994). However under water stress treatments we did not observe an increase in the concentration of these proteins.

On the contrary, the dehydrins showed a different behavior, as the bands did not appear in control leaves but they did in stressed leaves in all genotypes (Figure 6) and the increased expression was higher in leaves with lower RWC (Figure 6). Western Blots showed that dehydrins are proteins of about 25 kD, and although they were detected in plant leaves that have not been watered during 10 days, the band was stronger in those which had not been watered during 20 and 27 days (Figure 6).

Western blot did not show differences in dehydrin patterns between genotypes, showing an increased band in all lines in the second harvest and this band did not disappear in rewatered plants (Figure 6). Different authors observed an increase of dehydrin concentration as response to water stress that could be associated with difference in drought tolerante (Cellier *et al.* 1998; Lopez et al. 2003). This autor also showed that wheat cultivar less susceptible to drought expressed dehydrins with a higher leaf water potential. However, in our experiment, accumulation between genotypes was observed, and this response could not be related to the different RWC of genotype 2 under low water availability.

Since the tested genotypes behaved in a similar way under water stress, no difference in relation to induced gene expression could be established. However we work with two genes which expression under water stress was different. Some authors suggest that there are two groups of genes, a group that is expressed under water stress and another with ABA (Skriver and Mundy, 1990). It is possible that rob5 responds to ABA in contrast to the dehydrin gene.

Acknowledgments

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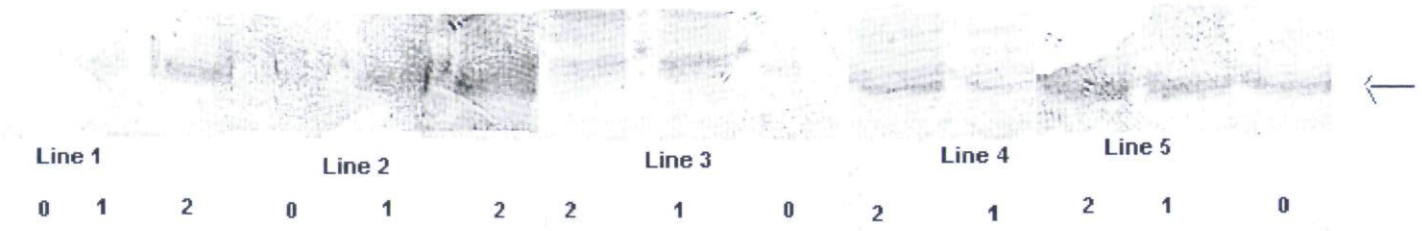

Figure 4. Western Blot analysis of Rob 5 antibody in five wheat genotypes. Leaves excised of well watered plants were dehydrated on the bench. We took samples for inmunoblot analysis at the start of the experiment (Time 0), 2 hours (Time 1) and 5 hours later (Time 2). Experiment I. Arrow indicates the band of Rob 5 protein.

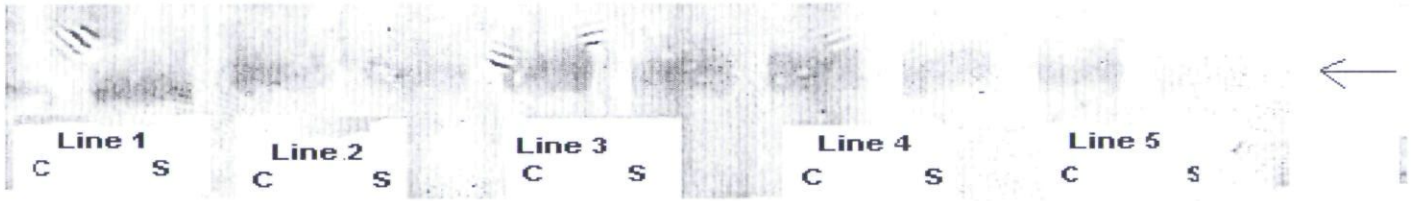

Figure 5. Western Blot analysis of Rob 5 antibody. Total protein from leaves were isolated from leaves of control (C) and stressed (S) wheat plants (after 14 days withholding water) Experiment II. Arrow indicates the Rob 5 band.

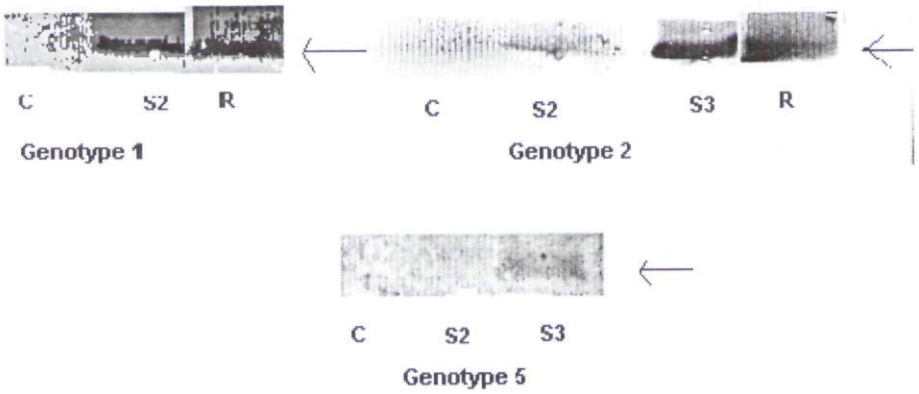

Figure 6. Western-Blot analysis of dehydrin antibody for genotype1, 2 and 5 on control plants (C) and stressed plants, second sampling date 14 days after withholding water (S2), 20 days after withholding water (S3) and rewatered plants after 27 days of withholding water (R). Experiment II. Arrow indicates dehydrin band

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