DROUGHT STRESS INCREASES THE EXPRESSION OF WHEAT LEAF RIBULOSE-1, 5- BISPHOSPHATE CARBOXYLASE/OXYENASE PROTEIN[*]

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Abstract – The effects of drought stress and exogenous abscisic acid on the expression of ribulose-1, 5-bisphosphate carboxyIase/oxygenase activase (Rubisco activase) were examined in wheat (*Triticum aestivum* L.). In response to water stress and abscisic acid, both the levels of endogenous abscisic acid and Rubisco activase increased in the leaves. Immunoblot analysis showed that both drought stress and abscisic acid, increased the amount of a 51KD activase form of the enzyme. In addition, our results from wheat leaves showed that Rubisco activase up regulated under drought stress by increasing the amount of endogenous abscisic acid. Therefore the level of Rubisco activase was dependent on the abscisic acid level. These results are consistent with previous findings that Rubisco activase increases under high temperature stress. These results indicate that the increased rate of activases synthesis may play a role in coping with the decline in photosynthetic capacity under drought stress. Effects of drought stress and abscisic acid and their interaction were highly significant (p<0.01) for all variables.

Keywords – Rubisco activase, drought stress, ABA treatment, protein expression, *Triticum aestivum* L

1. INTRODUCTION

Ribulose-1, 5-bisphosphate carboxy lase/oxygenase (Rubisco) is a key enzyme that initiates both photosynthetic and photorespiratory carbon metabolism. However to become catalytically active, Rubisco has to be first activated and carbanylated.

Ribulose-1, 5-bisphosphate carboxylase activase (Rubisco activase) can alter the activity of Rubisco by facilitating the dissociation of tightly bound sugar-phosphates from Rubisco in a process that requires ATP hydrolysis [1].

Drought can cause both reversible and irreversible inactivation of Rubisco. However, a complementary enzyme Rubisco activase release of tight binding inhibitors from Rubisco, which is itself regulated by redox controls [2].

Today, drought and temperature extremes are the major limitations for agronomic yield and crop quality. Wheat and corn are the major economic crop species, whose sensitivity to water stress is the main cause of annual crop losses [2].

Rubisco activase has become of interest to many agricultural crop investigators because the enzyme may be a means to improve the net rate of photosynthesis [1].

Abscisic acid (ABA) plays a crucial role in higher plants in their response to various environmental stresses acting as a regulatory link between stress factors and plant responses [3]. Physiological studies and molecular analysis have demonstrated that ABA may regulate the adaptation of plants to environmental stresses [4]. The increase in endogenous ABA concentration under water stress and the

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application of exogenous ABA is often accompanied by the expression of a number of specific proteins in plant tissues [5, 6].

Water deficit stress leads to significant changes in gene expression, leading to both increases and decreases in expression levels of specific genes. A number of different, and potentially signal transduction pathways regulate these changes in protein expression [7, 8]. ABA has been shown to be involved in the regulation of many stress-induced gene expressions in response to water deficit stress [9, 10].

The objective of the present study is to test the hypothesis that ABA- induced dehydration tolerance is related to the change of a protein that affects photosynthesis. The relationship between drought stress and ABA- pretreatment on the protein pattern of wheat has been investigated. These data suggest that both drought stress and ABA pretreatment increase Rubisco activase expression, which is a protein involved in photosynthesis.

2. MATERIALS AND METHODS

a) Plant Material

Wheat plants were grown in growth chambers at $25\,^{\circ}C/20\,^{\circ}C$ day/night temperatures, 65-70% R. H. and 16h/8h light-dark periods. Plants with fully expanded third leaves (5 weeks after planting) were used as experimental material [11].

Drought stress was imposed by withholding the water supply over a period of 14 days [12]. Each treatment was subjected to an analysis of variance (ANOVA) with drought stress and exogenous ABA treatment as the factors. Three replicates were used for each treatment.

b) ABA Pretreatment

A stock solution of 30 μm ABA was prepared in 0.4% ethyl alcohol [6]. For ABA treatments, leaves were soaked in an ABA stock solution for 2h and plants were then kept in the growth chamber for a period of 14days [13].

c) Leaf Protein Extraction

One gram of fresh leaf tissue was homogenized with 5 ml of extraction buffer containing 0.1M Tris-HCl, pH 7.4 at $4\,^{\circ}C$. The homogenate was centrifuged at 29000 g for 15 min and supernatant fluids stored at $-20\,^{\circ}C$. The amount of protein was determined by a Bio-rad protein assay using bovine serum albumin as standard [6]. All protein extraction steps were carried out at $4\,^{\circ}C$ unless otherwise indicated.

d) Gel Electrophoresis

Proteins were analyzed by electrophoresis on 10% SDS polyacrylamide slab gels, according to the method of Laemmli [14]. Gels were run at 25 mA constant current and stained with coomassie brilliant blue G. The proteins molecular masses were estimated with respect to the mobilities of the standard proteins (Sigma) [15].

e) ABA Analysis

Frozen leaf samples were lyophilized and cut finely with scissors. ABA was extracted with 4 mL extracting methanol using 15 mg of tissue in a 15 mL polypropylene tube. Samples were stirred overnight in the dark at $4\,^{\circ}C$. ABA was quantified by immunoassay using a polyclonal antibody to (S)-(+)- ABA [16].

f) Gel Filteration

The leaves protein extract was applied to sephacryl S-200 column (1cm*130cm) pre-equilibrated with PBS buffer (phosphate buffer –0.01M phosphate, 0.15M NaCl, pH 7.4) and the column was eluted with 0.1M Tris- HCl.

g) Western Blot

Proteins (30 μg) separated by 10% SDS- PAGE were transferred to a nitro cellulose membrane using a mini trans-blot electrophoresis transfer cell.

Nonspecific binding sites were blocked by 3% gelatin in PBS (phosphate buffer with NaCl and KCl, pH 7.4) buffer for 3h at room temperature.

The nitrocellulose membrane was incubated for 2h at room temperature with a primary antibody (rabbit anti–rubisco activase, polycolnal antiserum against recombinant cotton Rubisco activase was produced in rabbits using a commercial facility) [11].

The membrane was prewashed with PBS and then was incubated with goat anti-rabbit IgG alkaline phosphatase conjugate and detected using nitroblu-tetrazolium chloride (NBT) and bromo-4-chloro -3-indolyl-phosphate (BCIP) [6].

h) Protein Identification

For protein identification by LC/MS/MS, the Mascot MS/MS (Matrix science, London, UK) database searching program was used. Masco was used to search the MSDB database. High scores (>42 for peptides) in the database search indicated a likely match, confirmed or qualified by operator inspection of the spectra and search results.

3. RESULTS

To determine the effects of dehydration and ABA- pretreatment on protein synthesis, wheat leaves were fully extracted, and then the proteins were fractioned by SDS- PAGE. A protein band with a molecular weight of 51 KD appeared under both drought stress and exogenous ABA pretreatment. Such a band was absent in the control (Fig. 1). In general, there were major differences between the bands produced by drought stress and by ABA pretreatment.

Figure 1 shows the relative increase in protein content in leaves after ABA pretreatment and drought stress.
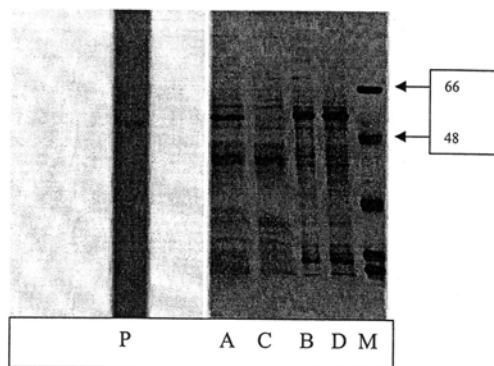

Fig. 1-10% SDS-PAGE gel electrophoresis of crude extract of wheat. M. molecular weight markers; Lane A, under drought stress. Lane C, control Lane B, under drought stress and exogenous ABA. Lane D, under exogenous ABA. Lane P, Rubisco activase eluted from sephacryl S-200 column

For purification and identification of the protein, plant total extracted protein was fractionated by gel filtration. It was applied to a column of sephacryl S-200 (Fig. 2). The fractions containing the protein (fractions 6 and 7) were combined, concentrated and using SDS gel electrophoresis, its molecular weight was estimated to be 51 KD. The electrophoresis profiles also demonstrated the purity of the protein. Occasionally, a minor extra peak of 47 KD was also observed following SDS-PAGE gel. As shown in Fig .2, the purified protein also showed a single band with a molecular weight of 51 KD by SDS-PAGE under both ABA and drought stress treatment.
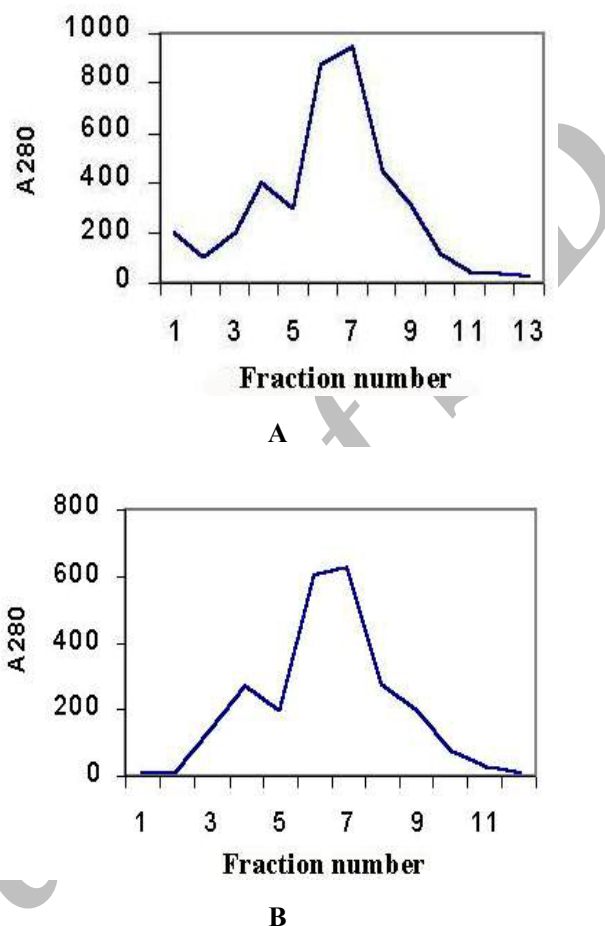

Fig. 2. Purification of Rubisco activase from total protein extraction (50mg loaded) of wheat leaf. The detailed experimental procedures are described in material and methods. ABA (A), drought + ABA (B)

Protein identification by LC/MS/MS analysis using Q-TOF MS (Performed by Australian Proteome Analysis Facility (APAF) relies on sequence information from the Mascot (matrix science, UK) database. Mascot was used to search the MSDB database. High scores (>42 for peptides in Mascot) in the database search indicated a likely match confirmed or qualified by operator inspection of spectra and search results.

The result summary for most likely matches follow, with identification given and all sequences shown with the N-terminals of the peptide on the left. The results of identifying show peptides of protein have homology with Rubisco activase barley. Both drought stress and ABA pretreatment have the same effect on endogenous ABA and Rubisco activase (Table 1).

The protein expression in leaves was increased under water stress and exogenous ABA treatments, so it is thought to be dependent on endogenous ABA. The leaves of the plant showed only one visible protein band that was immunologically related to Rubisco activase in the western blot (Fig. 3).

Table 1. Mean ($\pm$ SE) content of wheat leaf under water stress and ABA- pretreatment

Treatment	ABA(ng/mg)
Water stress	38.46 $\pm$ 0.90c
Water stress+ABA	56.06 $\pm$ 0.75b
+ABA	109.84 $\pm$ 0.82a
Control	6.07le
Mean	75.19
C.V.	2.3

Data is average of 3 independent replicates
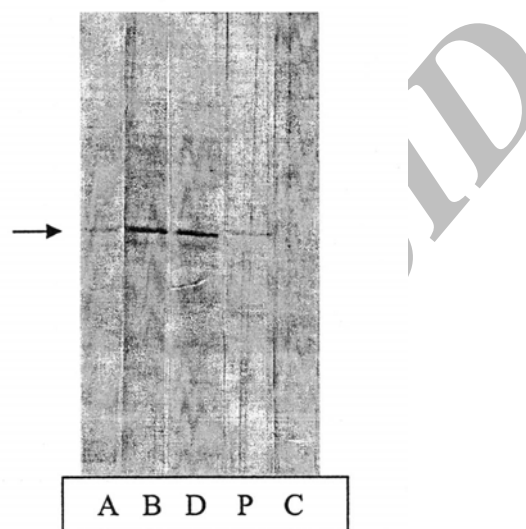Significant at the 0.01 level of probability

Fig . 3. Immunoblot analysis of wheat leaf Rubisco activase. Proteins were extracted from wheat leaves under drought stress). A), ABA- pretreatment and drought stress (B), ABA- pretreatment (D), control (C), purified protein eluted from sephacryl S-200(P). SDS-PAGE was followed by immunobloting onto nitrocellulose and detection using anti-crude immune serum and an alkaline phosphaltase conjugated secondary antibody. 30 micrograms of sample were loaded per lane

4. DISCUSSION

Taken together, these results indicate that increased synthesis of Rubisco activase under drought stress, exogenous ABA application and elevated levels of endogenous ABA may play a role in relieving the decline in wheat plant photosynthetic activity under these conditions [17, l8].

This conclusion is supported by previous reports demonstrating the markedly different thermal stabilities of activase isoforms from spinach [19] and the Rubisco activase acclimates in situ to high temperature when the stress is imposed at a slow rate, as might be expected to occur in the field. It has also been shown that Rubisco activase expression increases under high temperature in wheat [19]. We conclude that an altered expression of Rubisco activase might be crucial for continued CO_2 fixation under drought stress [11], protecting the plant photosynthetic capacity. The release of tight binding inhibitors requires the participation of Rubisco activase and hydrolysis of ATP [20, 21]. The removal of inhibitors by Rubisco activases may be impaired because of low ATP produced by drought [22]. In addition, Rubisco activase is susceptible to high temperatures [12, 23], and in this study it has been observed that Rubisco activase is susceptible to drought stress and exogenous ABA (Figs. 1 & 3), both of which increase endogenous ABA levels (Table 1) that may be associated in plant tolerance against water deficit.

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