**KMITL Sci. Tech. J. Vol. 7 No. S1 Nov. 2007**

# 18 kDa PROTEIN ACCUMULATION IN SUGARCANE LEAVES UNDER DROUGHT STRESS CONDITIONS

Nisachon Jangpromma[1], Supansa Kitthaisong[1], Sakda Daduang[1], Prasit Jaisil[2] and Sompong Thammasirirak[2]*

[1]Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen, 40002, Thailand
[2]Department of Agronomy, Faculty of Agriculture, Khon Kaen University, Khon Kaen, 40002, Thailand

## ABSTRACT

The alteration of protein synthesis or degradation is one of the fundamental metabolic processes that affects drought tolerance. To investigate the protein profiles of sugarcane exposed to drought stress, two unrelated lines of sugarcane (*Saccharum officinarum* L.) Khon Kaen 1 and K86-161were subjected to progressive water stress for 20 days. Under progressive drought stress condition, the Khon Kaen 1 line is sensitive to water stress. The modification of leaves were observed 2 weeks after the onset of water deficit. The Khon Kaen 1 leaves gradually turned yellow and wilted whereas the K86-161 leaves from the tolerant line remained green. The relative water content (RWC) in Khon Kaen 1 was decreased to 60% on day 20$^{th}$ whereas the RWC of K86-161 was slightly decreased and remained constant at 86% upon exposure to the water stress. In addition, when the protein changed in leaves were studied by two-dimensional electrophoresis, the accumulation of a 18 kDa protein was detected in drought-tolerant, K86-161 line. Antiserum raised against this protein, purified from SDS-PAGE, was used as a probe to detect the protein in sugarcane leaves by Western blotting technique. The titer of antigen-antibody interaction, was estimated to be 1:100. Furthermore, this polyclonal antibody was used to detect the accumulation of the 18 kDa protein in K86-161 and Khon Kaen 1 sugarcane leaves by Western blotting technique. The results show that the 18 kDa protein band from K86-161 expressed higher intensity than that of Khon Kaen 1 in equivalent to total protein amount. These results indicate that 18 kDa protein expressions had a high potential for development as a marker in any screening technique for drought-tolerant sugarcane cultivars.

**KEYWORDS:** sugarcane, drought tolerance, polyclonal antibody, western blotting technique

*Corresponding author: Tel: +66-43-342911 Fax: +66-43-342911
E-mail: somkly@kku.ac.th

## 1. INTRODUCTION

Sugarcane is a major industrial crop cultivated in tropical and sub-tropical regions to produce sugar. The sugarcane has been cultivated either in arable or drought region [1-3]. However, sugarcane production in the Northeastern Thailand still faces some problems that should be resolved. One of the problems is low productivity of recent sugarcane lines according to poor ratooning ability. This problem leads to the high cost production. To overcome this problem, the analytical method to detect the drought tolerance sugarcane lines should be developed for planting programme.

Abiotic stresses can directly or indirectly affect the physiological status of an organism by altering its metabolism, growth, and development. A common response of organisms to drought, salinity, and low-temperature stresses is the accumulation of sugars and other compatible solutes. These compounds serve as osmoprotectants and, in some cases, stabilize biomolecules under stress conditions [4-6]. Drought stress is a major environmental factor affecting the sugarcane productivity in the Northeastern Thailand. It induces accumulation of compatible solutes, such as proline, sugar, sugar alcohol and betaine in plants [7-8]. These compounds help plants to adapt to the severe condition by means of specific biochemical functions. A specific behavior is also observed in the aerial part of the plant: the first leaves, developed before the water stress, wilt progressively, whereas the leaves that emerge after the onset of water deficit remain turgid and harden [9].

Biomarkers to identify and characterize the drought-tolerant sugarcane lines have been sought for many decades. These markers fall under three broad categories: morphological, cytogenetic, and biochemical. Morphological markers are based on the traditional botanical descriptions of visible characters and were the first markers to be utilized. They are of limited value because in sugarcane they are not inherited in a simple Mendelian manner [10]. Cytogenetic markers observed from mitotic and meiotic chromosomes provide additional information, but the small and variable size of sugarcane chromosomes and their abnormal pairing behavior make difficultly cytogenetic observations [11]. Biochemical markers became a popular tool in plant genetics, and studies utilizing such markers were also initiated in sugarcane [12]. Protein and secondary metabolites of leaves from variety of plants have been examined and were found to be promising as markers, such as dehydrin [13-15], superoxide dismutase [16], ASR (ABA-water stress-ripening-induced) protein [17], actin depolymerizing factor [18]. Thus, in this study, proteins involved in drought stress from sugarcane leaves were screened by proteomics. Furthermore, we used this protein to screen drought-tolerant sugarcane lines by Western blotting technique.

## 2. MATERIALS AND METHODS

### 2.1 Plants and drought stress condition

Two unrelated lines of sugarcane plants (K86-161 and Khon Kaen 1) were grown in a greenhouse for the evaluation of drought tolerance. In the greenhouse, the propagative organs of K86-161 and Khon Kaen 1 were grown in pots (50 cm in diameter and 30 cm deep) containing topsoil under natural light conditions. Soil had pH of 4.75 and contained total nitrogen 0.0255 %; field capacity (FC) of 12.26 % and permanent wilting point (PWC) of 3.36%. After growing for 3 months, the K86-161 and Khon Kaen 1 sugarcane plants were treated by stopping watering for 3 weeks. Leaf samples were collected from stressed and control plants after the drought treatments.

### 2.2 Protein extraction

Sugarcane leaves were ground to fine powder using liquid nitrogen and suspended in lysis buffer, which contain 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (w/v) DTT, 0.8% (w/v) IPG buffer (Amersham Bioscience, Sweden). The homogenate was centrifuged for 30 min at 10,000 xg at 4°C. The supernatant was transferred to a new 1.5 ml tube and cleaned to remove the contaminating substance by 2D-Clean up kit. The pellets were resuspended in rehydration buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2 mM (w/v) DTT, 0.8% (w/v) IPG buffer and 0.2% bromophenol blue. The protein concentration was determined by the Bradford assay with BSA as the standard.

### 2.3 Protein analysis by gel electrophoresis

One-dimensional polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously [19] using 15% separating gel and 4% stacking gel. Total proteins (0.05-0.1 μg) were loaded in each well and separated on a Mini-Protein III Cell (Bio-Rad, U.S.A) at 150 constant volts until the tracking dye reached the bottom of the gel. The following molecular weight standards (Amersham Bioscience, Sweden) were run alongside the extracted proteins: phosphorylase b, 97 kDa; albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; and α lactalbumin, 14.4 kDa. Proteins were stained with Coomassie brillant blue R-250. Two-dimensional (2-D) electrophoresis was performed according to a modified method of O'Farrell [20]. For first dimension isoelectric focusing gel, proteins were resuspended in the rehydration buffer. Samples were allowed to rehydrate at least 1 h on ice. Two hundred and fifty μg of proteins were loaded onto IPG strip (13 cm, pH 4-7, Amersham Bioscience, Sweden). The second dimension SDS gel contained 12.5% acrylamide. The molecular weight standards were as described previously. Following electrophoresis of the second dimension, proteins were stained with silver nitrate.

### 2.4 Production of polyclonal antibody

Sample containing total proteins (500 μg) were separated by SDS-PAGE and the bands of protein related to drought stress were excised from the gels. The lyophilized protein bands were ground to fine powder and suspended in 1 to 2 ml of PBS buffer (135 mM NaCl, 1.5 mM $KH_2PO_4$, 2.5 mM KCl, and 8 mM $Na_2HPO_4$). A suspension of protein related to drought stress and Freund's complete adjuvant (Sigma, USA) was sufficiently emulsified. One hundred μl of the emulsion was subcutaneously injected into mice. Two weeks following the second boost, the same emulsion but containing Freund's incomplete adjuvant (Sigma, USA) instead of the complete adjuvant was subcutaneously injected into mice at intervals over 8 weeks. Blood was drawn from the mice 2 weeks after the last injection and allowed to clot overnight at 4°C. The serum was collected by centrifugation at 8,000 xg for 10 min and then the supernatant containing anti-18 kDa protein antibody was pooled and used for Western blotting.

### 2.5 Western blotting analysis

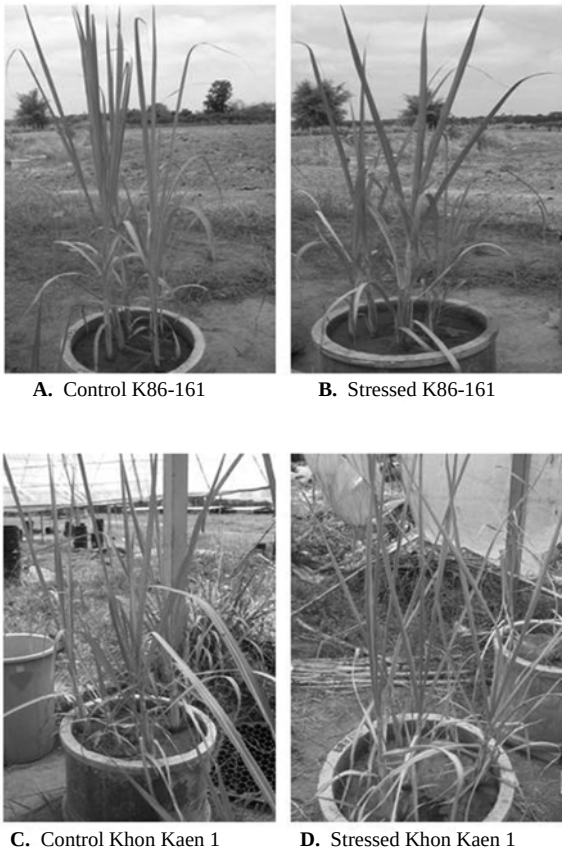
After separation by electrophoresis, proteins were transferred onto a nitrocellulose membrane (Bio-Rad, USA) in transfer buffer containing 48 mM Tris-base, 39 mM glycine buffer containing 20% methanol at a constant current of 130 mA for 1 h. The membrane was incubated in blocking solution (5% skimmed milk in TBST buffer) at room temperature for 1 h. Next, the membrane containing proteins was incubated with the first antibody diluted 1:100 with blocking solution at room temperature for 1 hrs. After rinsing the membrane three times for 5 min each with TBST (10 mM Tris–HCl, 150 mM NaCl containing 0.05% Tween 20), the membrane was incubated in alkaline phosphatase-goat antimouse IgG (1:500 in TBST) for 1 hrs. at room temperature. The membrane was again rinsed three times for 5 min each with TBST, followed by TBS pH 8.0 (10 mM Tris–HCl containing 150 mM NaCl) three times, and then substrate buffer, pH 9.5 (100 mM

Tris-base, 100 mM NaCl containing 50 mM $MgC_{l2}{\cdot}6H_2O$) twice for 30 s each at room temperature. Visualization of any immunoreaction was carried out by incubating the membrane in a substrate solution containing 30 µl *p*-nitroblue tetrazolium chloride (NBT) and 30 µl 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 5 ml substrate buffer, pH 9.5 for at least 5 min. The color reaction was stopped by transferring the membrane into water. Control membrane with marker was stained with 0.1% amido black.

## 3. RESULTS AND DISCUSSION

### 3.1 Plant responses to drought stress

To study and determine the drought tolerance of sugarcane lines, 3-month-old K86-161 and Khon Kaen 1 sugarcane plants were drought stressed under non-watering conditions for 20 days. Various degrees of wilting were observed after drought treatment. After stress treatment, the leaves of Khon Kaen 1 line became wilted and yellow. In contrast, K86-161 showed normal growth and morphology until the end of experiment (Figure 1).

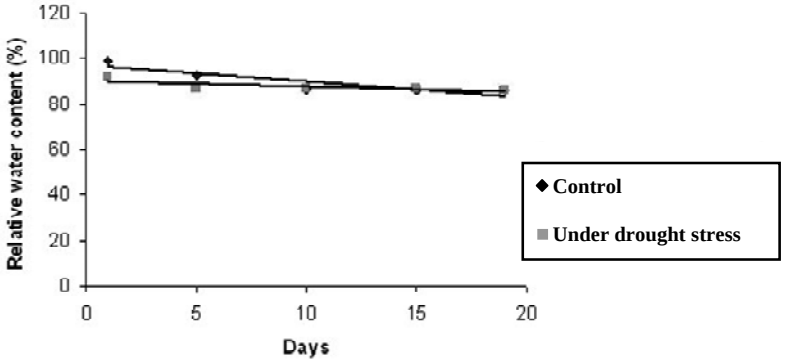


**A.** Control K86-161 **B.** Stressed K86-161

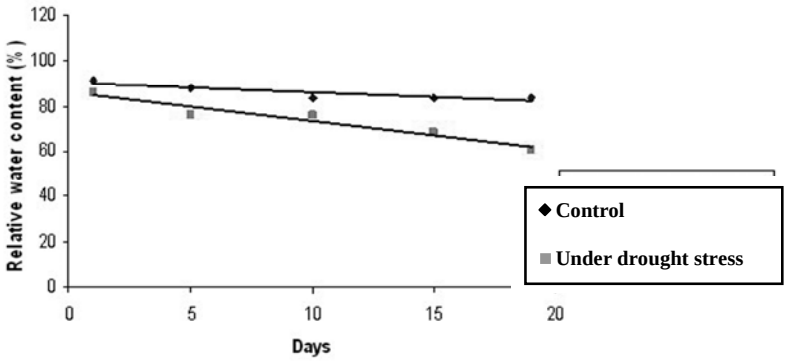
**C.** Control Khon Kaen 1 **D.** Stressed Khon Kaen 1

**Figure 1** Sugarcane was grown in pots under drought condition. A. Control K86-161, B. Stressed K86-16, C. Control Khon Kaen 1 and D. Stressed Khon Kaen 1.

Changes in relative water content (RWC) of plant tissues can well reflect the effects of water stress on plants [21]. When the K86-161 and Khon Kaen 1 sugarcane lines were grown in the pots for 3 months, RWC of leaves were measured to be about 92% and 86% respectively. RWC was used to determine drought stress condition. Figure 2 showed that at the day start, no significant different in RWC of both sugarcane lines. However, after 10 days, the morphology change, wilting showed in Khon Kaen 1. The RWC of stressed Khon Kaen 1 reduced to 75%. At 20 days, the RWC of stressed Khon Kaen 1 dramatically decreases to 60%, whereas stressed K86-161 still maintains 86% RWC. Moreover, the leaves of stressed Khon Kaen 1 shows obvious wilted comparing to leaves of K86-161. From this result we conclude that K86-161 might be a drought stress tolerance line.




**A.** RWC of K 86 -161




**B.** RWC of Khon Kaen 1

**Figure 2** Effect of drought stress on relative water content (RWC) of sugarcane leaves A. Relative water content of K86-161, B. Relative water content of Khon Kaen 1.

### 3.2 Comparison of protein patterns of K86-161 and Khon Kaen 1 under drought stress

The effect of drought stress on protein expression patterns in sugarcane leaves were examined by treating sugarcane plants with withholding watering. After the treatment, proteins from leaves of both sugarcane lines were extracted and separated by 2D-PAGE. Silver-stained gels displayed more than 500 protein spots in K86-161 and more than 400 protein spots in Khon Kaen 1 (Figure 3). There are many proteins in K86-161, 128 protein spots showed a response to water deficit. Of these proteins, 54 protein spots were only detected in response to drought stress compared to control. Forty-six protein spots were up-regulated and 28 protein spots were down-regulated. In Khon Kaen 1 line, 104 protein spots showed a change in abundance under stress; of these 30 protein spots were only detected in response to drought stress. Forty-two protein spots were up-regulated and 32 protein spots were down-regulated. Drought-induced polypeptides have been observed in many studies [22-24] Mostly; found polypeptide play a role in water stress tolerance. Our results in sugarcane also indicated that among the modifications induced by drought; up-regulated of a highly intense 18 kDa protein was particularly noticeable and it can be possibly used as protein marker (Figure 4). In the 2D-patterns from drought-stressed leaves, the intensity of a 18 kDa protein spot was increased when compared to the control leaves of K86-161, which is a drought-tolerant sugarcane line (Figure 4, A and B). In contrast, the increase of 18 kDa protein in the leaves of sensitive line Khon Kaen 1 was decreased when compared to control (Figure 4, C and D). The relationship between 18 kDa protein changes and drought-tolerant was still to be unknown. However, previous study [24] also found that the accumulation of 18-, 28-, 31-kDa dehydrin-like proteins in the seeds of crowfoot (*Ranunculus sceleratus* L.) depended on stages of water stress. In bromegrass (*Bromus inermis* Leyss) the polypeptide with a calculated molecular mass of 18.08 kDa and isoelectric point (pI) of 7.50 was induced by ABA, cold and drought stress [25]. So, the accumulation of an 18 kDa protein was responsive to drought stress and may play important role to preserve water in plant.

### 3.3 Accumulation of 18 kDa protein in sugarcane leaves

Accumulation of the 18 kDa protein in sugarcane leaves was investigated by Western blotting. The 18 kDa protein was purified by SDS-PAGE. The bands of this protein were excised from the gels and used as immunogen for antibody production in mice. The titer of antigen-antibody interaction at around 1:100 was obtained. The polyclonal antibody was used to detect drought tolerant sugarcane line (K86-161) and drought intolerant sugarcane line (Khon Kaen 1) by Western blotting technique. Both plants were grown in the field under natural drought condition. The result showed that the 18 kDa protein band from K86-161 expressed higher intensity than that of Khon Kaen 1 in equivalent total protein amount (Figure 5). These results indicated that the 18 kDa protein expressions had a high potential for development as a marker in any screening techniques for drought-tolerant sugarcane lines.

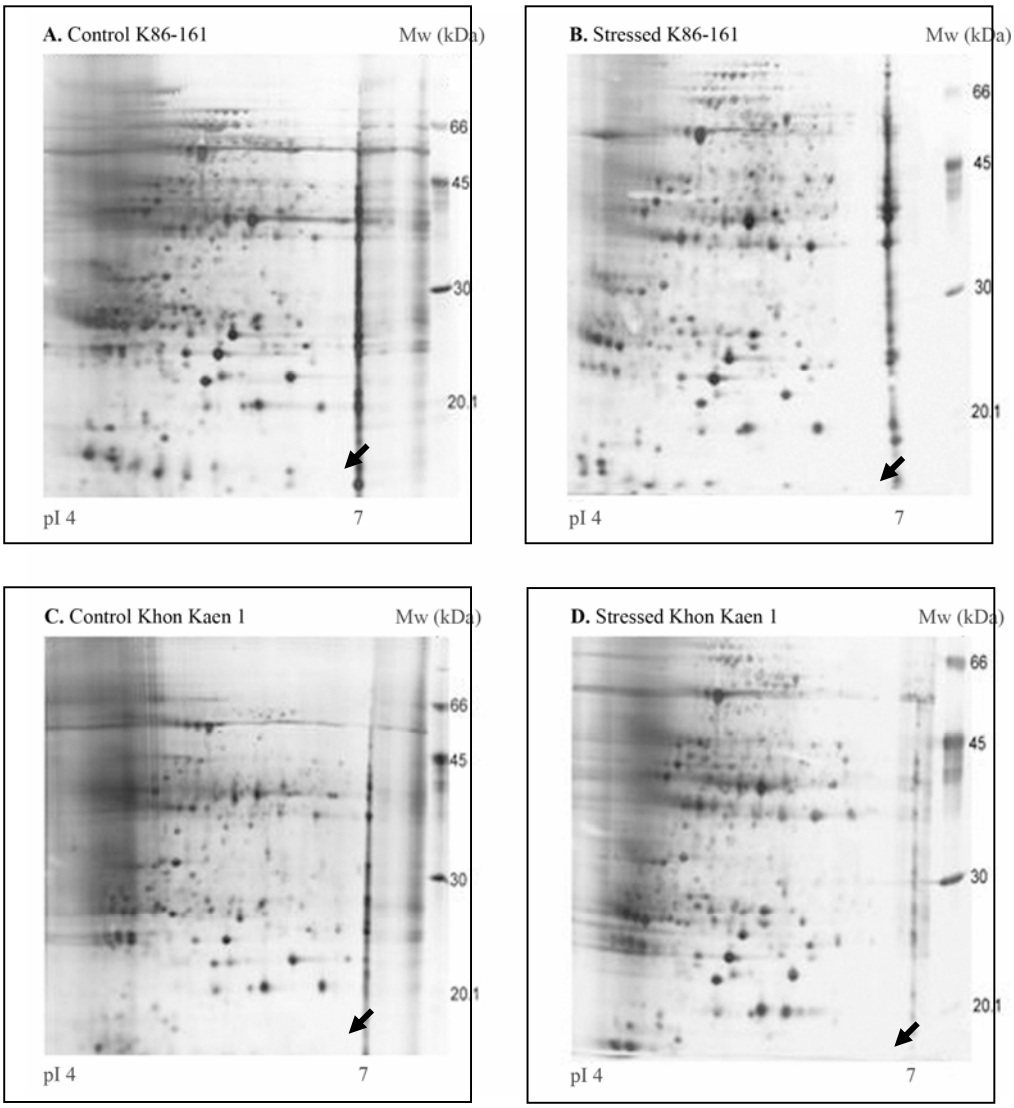



**Figure 3** Protein patterns in sugarcane leaves exposed to 20 days of drought stress. Proteins were extracted from leaves, separated by 2D-PAGE. In the first dimension (IEF), 250 µg of protein was loaded onto a 13 cm IPG strip with a linear gradient of pH 4-7. In the second dimension (SDS-PAGE), 12.5 % polyacrylamide gel was used. Proteins were visualized by silver staining. A. Control K86-161 (well-watered plants),B. Drought-stressed K86-161,C. Control Khon Kaen 1 (well-watered plants), D. Drought-stressed Khon Kaen1. The arrows point to 18 kDa protein position.

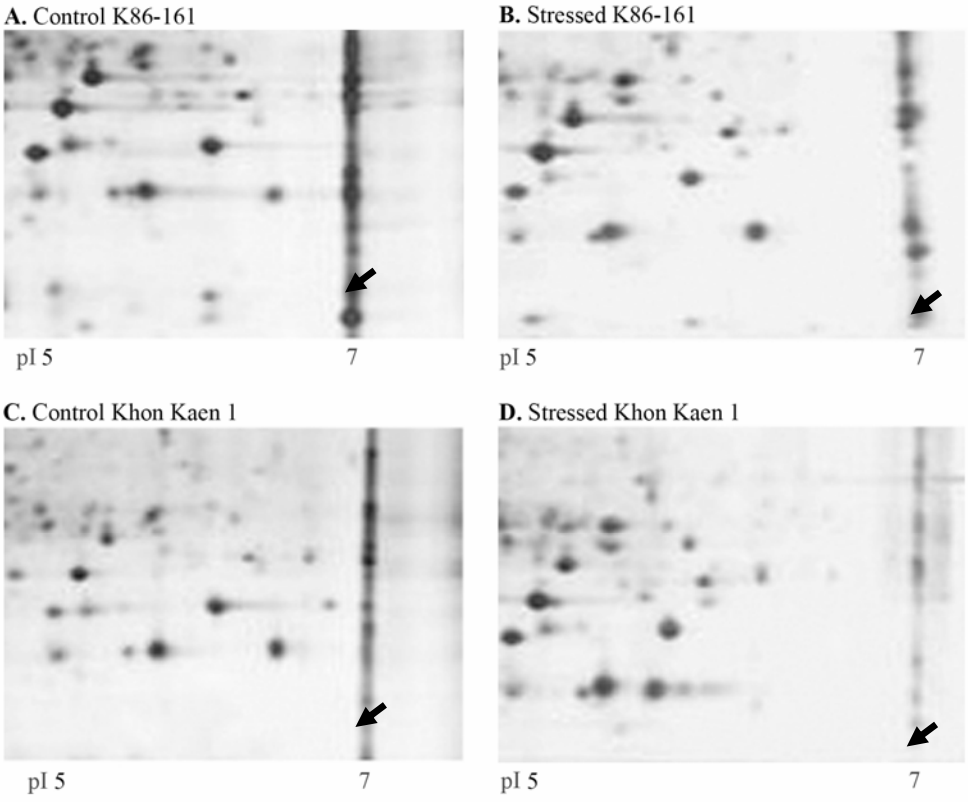



**Figure 4** Silver stained 2D-gel of protein from sugarcane leaves. A. Control K86-161 (well-watered plants), B. Drought-stressed K86-161,C.Control Khon Kaen 1 (well-watered plants), D. Drought-stressed Khon Kaen1. The arrows point to 18 kDa protein position.

## 4. CONCLUSIONS

In conclusion, under water stress conditions, the leaves of Khon Kaen 1 sugarcane line wilted and turned yellow. In contrast to K86-161 showed normal growth when compared to the control. Relative water content (RWC) in the leaves of stressed Khon Kaen 1 decreased from 83% to 60% in 20 days, while it stayed at 84 % in those of the controls. This stress can be considered quite severe for sugarcane plants, since RWC of leaf lower than 70% is known to induce marked damages. In K86-161 leaves, RWC decreased slightly and remained constant at 86% in both controlled and stressed plants. These results indicated that K86-161 was more tolerant to water stress than Khon Kaen 1.

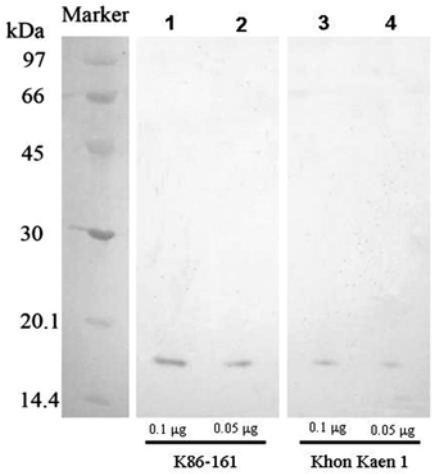



**Figure 5** Western blotting analysis of crude protein extracts from sugarcane leaves using the polyclonal antibody raised against 18 kDa proteins (dilution of antiserum, 1:100). The bands were visualized by alkaline phosphatase reaction. Lanes: 1 and 2 crude protein extracts from sugarcane leaves of K86-161 line 0.1 and 0.05 µg/well respectively, Lanes: 3 and 4 crude protein extracts from sugarcane leaves of Khon Kaen 1 line 0.1 and 0.05 µg/well respectively. Both plants were grown in the field under natural drought condition.

After 2D-PAGE separation, the accumulation of 18 kDa protein was found in K86-161 sugarcane plant under drought stress conditions. The intensity of this protein was increased in drought stressed K86-161 which is the drought tolerant sugarcane line when compared to control and both conditions (well-watered and drought stressed plants) of Khon Kaen 1 which is the sensitive sugarcane line. Furthermore, the polyclonal antibody was raised against 18 kDa protein and used for Western analysis. The result showed that the 18 kDa protein bands from K86-161 expressed higher intensity than that of Khon Kaen 1 in equivalent total protein amount, thus confirming the accumulation of the 18 kDa protein in response to water deficit and it has a high potential for development as a marker in any screening techniques for drought-tolerant sugarcane lines.

## 5. ACKNOWLEDGEMENTS

This study was financially supported by Agriculture Biotechnology Project and Agriculture Biotechnology Research Center for Sustainable Economy at Khon Kaen University. We thank Khon Kaen Field Crop Research Center for providing the sugarcane cultivars.

## REFERENCES


[1] Zhang, S.Z. *et al*. **2006** Expression of the *Grifola Frondosa* Trehalose Synthase Gene and Improvement of Drought-tolerance in Sugarcane (*Sacchrum officinarum* L.), *Journal of Integrative Plant Biology, 48*(4), 453-459.

[2] Sugiharto, B. **2004** Biochemical and Molecular Studies on Sucrose-phosphate Synthase and Drought Inducible-protein in Sugarcane (*Saccharum officinarum*), *Journal ILMU DASAR, 5*(1), 62-67.

[3] Inman-Bamber, N.G. and Smith, D.M. **2005** Water Relations in Sugarcane and Response to Water Deficits, *Field Crops Research, 92*, 185-202.

[4] Yancey, P., Clark, M., Hand, S., Bowlus, R. and Somero, G. **1982** Living with Water Stress: Evolution of Osmolyte Systems, *Science, 217*, 1214-1222.

[5] Hare, P.D., Cress, W.A. and Staden, J. **1998** Dissecting the Roles of Osmolyte Accumulation During Stress, *Plant, Cell and Environment, 21*, 535-553.

[6] Zhang, S. Z., Yang, B. P., Feng, C. L. and Tang, H. L. **2005** Genetic Transformation of Tobacco with the Trehalose Synthase Gene from *Grifola frondosa* Fr. Enhances the Resistance to Drought and Salt in Tobacco, *Journal of Integrative Plant Biology, 47*(5), 579-587.

[7] Rhodes, D. and Hanson, A. **1993** Quaternary Ammonium and Tertiary Sulfonium Compounds in Higher Plants, *Annual Review of Plant Physiology and Plant Molecular Biology, 44*, 357-384.

[8] Ingram, J. and Bartels, D. **1996** The Molecular Basis of Dehydration Tolerance in Plants, *Annual Review of Plant Physiology and Plant Molecular Biology, 47*, 377-403.

[9] Reviron, M. P., Vartanian, N., Sallantin, M., Huet, J. C., Pernollet, J. C. and de Vienne, D. **1992** Characterization of A Novel Protein Induced by Progressive or Rapid Drought and Salinity in *Brassica napus* leaves, *Plant Physiology, 100* (3), 1486-1493.

[10] Hogarth, D. M. **1987** Genetics of Sugarcane. In: Heinz, D. J., Ed. *Sugarcane Improvement Through Breeding.* Amsterdam, Elsevier, pp. 255-271.

[11] Sreenivasan, T. V., Ahooliwalia, B. S. and Heinz, D. J. **1987** Cytogenetics. In: Heinz, D. J., Ed. *Sugarcane Improvement Through Breeding*. Amsterdam, Elsevier, pp. 211-253.

[12] Ramagopal, S. **1990** Protein Polymorphism in Sugarcane Revealed by Two-dimensional gel Analysis. *Theoretical and Applied Genetics, 79*, 297-304.

[13] Lopez, C.G., Banowetz, G.M., Peterson, C.J. and Kronstad, W.E. **2003** Dehydrin Expression and Drought Tolerance in Seven Wheat Cultivars, *Crop Science, 43*, 577-582.

[14] Jiang, Y. and Huang, B. **2002** Protein Alterations in Tall Fescue in Response to Drought Stress and Abscisic Acid, *Crop Science, 42*, 202-207.

[15] Lopez, C.G., Banowetz, G.M., Peterson, C.J. and Kronstad, W.E. **2001** Differential Accumulation of A 24-kd Dehydrin Protein in Wheat Seedlings Correlates with Drought Stress Tolerance at Grain Filling, *Hereditas, 135*, 175-181.

[16] Zang, X. and Komatsu, S. **2007** A Proteomics Approach for Identifying Osmotic-stress-Related Proteins in Rice, *Phytochemistry, 68* (4), 426-437.

[17] Riccardi, F., Gazeau, P., de Vienne, D. and Zivy, M. **1998** Protein Changes in Response to Progressive Water Deficit in Maize. Quantitative Variation and Polypeptide Identification, *Plant Physiology, 117* (4), 1253-1263.

[18] Salekdeh, G.H., Siopongco, J., Wade, L.J., Ghareyazie, B. and Bennett, J. **2002** A Proteomics Approach to Analyzing Drought and Salt-responsiveness in Rice, *Field Crops Research, 76*, 199-219.

[19] Laemmli, U.K. **1970** Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4, *Nature, 227*(5259), 680-685.

[20] O'Farrell, P.H. **1975** High Resolution Two-dimensional Electrophoresis of Proteins, *The Journal of Biological Chemistry, 250*(10), 4007-4021.

[21] Havaux, M., Canaani, O. and Malkin, S. **1986** Photosynthetic Responses of Leaves to Water Stress, Expressed by Photoacoustics and Related Methods: I. Probing the Photoacoustic Method as An Indicator for Water Stress in Vivo, *Plant Physiology, 82*(3), 827-833.

[22] Arora, R., Pitchay, D.S. and Bearce, B.C. **1998** Water-stress-induced Heat Tolerance in Geranium Leaf Tissues: A Possible Linkage Through Stress Proteins? *Physiologia Plantarum, 103*, 24–34.

[23] Bewley, J.D., Larsen, K.M. and Papp, J.E.T. **1983** Water-stress Induced Changes in the Pattern of Protein Synthesis in Maize Seedling Mesocotyls: A Comparison with the Effects of Heat Shock, *Journal of Experimental Botany, 34*, 1126–1133.

[24] Perez-Molphe-Balch, E., Gidekel, M., Segura-Nieto, M., Herrera-Estrella, L. and Ochoa-Alejo, N. **1996** Effects of Water Stress on Plant Growth and Root Proteins in Three Cultivars of Rice (*Oryza sativa*) with Different Level of Drought Tolerance, *Physiologia Plantarum, 96*, 284–290.

[25] Wu, G., Robertson, A.J., Zheng, P., Liu, X. and Gusta, L.V. **2005** Identification and Immunogold Localization of a Novel Bromegrass (*Bromus inermis* Leyss) Peroxisome Channel Protein Induced by ABA, Cold and Drought Stresses, and Late Embryogenesis, *Gene, 363*, 77-84.