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Isolation and Identification of Peanut Leaf Proteins Regulated by Water Stress

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Abstract: Water deficits trigger signaling cascades leading to modulation of protein expression in plant tissues. Identification of peanut leaf proteins regulated by water stress provides some insights of cellular and molecular response of peanut plants to drought stress. Peanut variety Khon Kaen 4, a water-stress sensitive variety, was grown in a growth chamber under controlled environment. Water stress was imposed on day 30 after seedling emergence by withholding watering peanut plants for 6 days as compared to plants adequately supplied with water. Total protein were prepared from a leaflet of fully expanded leaf on the main stem. Proteins were separated in duplicated gels using two-dimensional gel electrophoresis and visualized by silver nitrate staining. Image analysis was performed using ImageMaster 2D Platinum 5.0 to determine proteins regulated by water stress. Molecular mass and isoelectric point of each regulated protein were used in database queries for protein identification. One protein was induced under water stress and the homologous protein was identified as Serine/threonine-protein phosphatase PP 1. Five proteins were down-regulated by water deficit. The homologous proteins were chaperone protein DNAJ, auxin-responsive protein IAA29, peroxidase 43, caffeoyl-CoA O-methyltransferase and SNF1-related protein kinase regulatory subunit beta-2. Down-regulated proteins may be associated with sensitivity of the peanut variety to water stress.

Key words: Peanut, water stress, two-dimensional gel electrophoresis, proteins

INTRODUCTION

Drought stress brings about physiological and morphological changes in peanut crop allowing the plant to maximize water uptake while minimizing water lost (Wright and Nageawara Rao, 1994). However, the cellular mechanism of responses in peanut plants to water stress is not well documented. Oxidative stress occurs in photosynthetic tissues as a result of water deficit and this will lead to the destruction of photosynthetic apparatus and other macromolecules within plant cells. Ion homeostasis and conformation of proteins are perturbed as plant cells lose more water and this will be detrimental to cellular activities (Bray *et al*., 2000; Xiong and Zhu, 2002). Plant cells are capable of dealing with water deficit by alteration of cellular activities at the level of gene expression. Under water stress conditions, signaling cascades are initiated leading to the activation or suppression of gene expression (Bray, 2004; Yamaguchi-Shinozaki and Shinozaki, 2006; Zhu, 2002). Some of the gene products are regulatory and functional proteins working in concert to cope with oxidative stress and cellular abnormalities (Bray *et al*., 2000; Valliyodan and Nguyen, 2006). The expression of some proteins is modulated including enzymes involved in biosynthesis of osmolytes (Legaria *et al*., 1998; Russel *et al*., 1998), carrier and channel proteins (Roberts, 1998; Vera-Estrella *et al*., 2004; Xiong and Zhu, 2002); enzymes responsible for scavenging reactive oxygen species (Mittler *et al*., 2004); enzymes involved in repairing and degrading damaged proteins (Xiong and Zhu, 2002); late embryogenesis abundant (Lea) proteins, heat shock proteins and molecular chaperones (Hong Bo *et al*., 2005; Xiong and Zhu, 2002); enzymes involved in lignin biosynthesis (Vincent *et al*., 2005). Identification of proteins up or down regulated by water deficits will help to provide some insights of cellular mechanisms of drought tolerance in crop plants. The objective of this study was to isolate and identify peanut leaf proteins regulated by water stress conditions.

MATERIALS AND METHODS

Plant materials: A drought-susceptible peanut variety, Khon Kaen 4, was grown in a phytotron climate simulator, (Contherm, Australia). Environmental conditions in the

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growth chamber were set as follows; irradiance 720 μ mol m^{-2} sec^{-1}, a light/dark regime of 12 h light and 12 h dark at 35/30°C, 85% relative humidity during light and dark hours. Peanut seeds were sown into 6 pots containing 9 kg air-dried soil. One week after emergence, seedlings were thinned to obtain two uniform seedlings per pot. Two weeks after seedling emergence, 0.5 g of 12-24-12 fertilizer was applied to each pot. On day 30 after seedling emergence, pots were divided into two groups; the first group for water stress treatment and the second group as control treatment. In the first set, water stress was created by withholding watering for 4 to 6 days while soil moisture of the control pots was maintained at field capacity. On days 4 to 6 after withholding watering, leaf samples were harvested and leaf water potential and relative water content were measured for both water stress and control treatments. The first fully expanded leaf on the main stem was harvested and frozen in liquid nitrogen and then stored at -80°C until protein extraction. The second fully expanded leaf was used for determining relative water content (Barrs and Weatherley, 1962). The third up to fifth fully expanded leaves on the main stem were used for leaf water potential determination (Tomos and Leigh, 1999).

Protein preparation and separation: A single leaflet from stressed and non-stressed plants on day 6 after withholding watering was ground in liquid nitrogen using mortar and pestle. Total proteins in the ground materials were extracted at 4°C in 2 mL of lysis buffer containing 7M urea, 2M thiourea, 4% (w/v) CHAPS pH 4-7, 60 mM DTT and 2% (v/v) IPG buffer (Amersham Biosciences) (Berkelman and Stenstedt, 1998). The protein extracts were centrifuged at 4°C for 20 min at 13,000x g and the supernatants were collected. Fifty microliter of the supernatant was cleaned using Clean Up Kit (Amersham Biosciences). The protein pellets were resuspended in 100 μL rehydration buffer containing 8M urea, 2% (w/v) CHAPS, 60 mM DTT, 0.1% (v/v) protease inhibitor and 2% (v/v) IPG buffer (pH 4-7) (Amersham Biosciences). Protein concentrations were measured using Quant Kit (Amersham Biosciences). Individual 13 cm IPG strips were rehydrated overnight with 250 μL rehydration buffer containing 30 μg proteins in a reswelling tray at 20°C. Isoelectric focusing was carried out at 20°C using Amersham Biosciences Multiphor II system equipped with cooling system and Amersham Biosciences 3500XL power supply. There were 3 phases of running conditions; 1 min at 300V, 1:30 h at 3,500V and 1:30 h at 3,500V. After electrofocusing, the strips were equilibrated in 2 steps with buffer solution containing 6 M urea, 30% (v/v) glycerol, 50 mM Tris-HCl, pH 6.8 and 2% (w/v) SDS; the buffer in the first step containing 1% (w/v) DTT and in the second step containing 2.5% (w/v) iodoacetamide (Berkelman and Stenstedt, 1998). After equilibration, proteins were separated in 12.5% SDS-PAGE according to their molecular sizes using Hoefer SE600 system. Proteins in each leaflet sample were separated in duplicated gels.

Spot detection: Proteins in gels were visualized by silver nitrate staining (Shevchenko *et al*., 1996). The gels were scanned with ImageScanner equipped with Labscan version 5.0 (Amersham Biosciences) at 600 dpi. Image analysis of gels was performed using ImageMaster 2D Platinum 5.0 (Amersham Biosciences). Protein spots in the gel were checked by visualization. The vague spots were individually visualized using 3D view tool and deleted if they were not well illustrated. Twelve protein reference spots were randomly selected and molecular mass (M_r) and isoelectric point (pI) were estimated using molecular mass markers (LMW Calibration Kit; Amersham Biosciences) as references. M_r and pI for the remaining proteins were calculated by the ImageMaster 2D Platinum 5.0. Differential expression of proteins in peanut leaves under adequate water supply and water stress was determined. Water-stress regulated proteins with M_r and pI were recorded.

Database queries and protein identification: Only proteins with percentage volume of 0.1 or greater were used in the database queries in order to avoid artifacts. M_r and pI were used for searching protein identities using TagIdent of the ExPasy tools (http://au.expasy.org/tools/tagident.html). The pI range of 0.1, molecular range of 5% and plant were used in the queries. Tissue specificity, induction, number of hits and protein function were also taken into account for protein identification.

RESULTS

Water status of plants: Relative water contents of non-stressed plants were between 94 and 96%. Withholding watering for 4 to 6 days caused a large reduction in relative water contents from 97 to 69% (Fig. 1A). Leaf water potential of plants receiving adequate water supply were between -0.06 and -0.09 MPa. Under stress conditions, leaf water potential declined from -0.06 MPa to -0.27 MPa (Fig. 1B).

Resolution of protein separation in two dimensional gels: Proteins were well separated into individual spots. Patterns of proteins in stressed and non-stressed plants are very similar in both replicates (Fig. 2 and 3). Protein spots were well recognized by the image analysis software and 12 reference spots could be assigned (Fig. 4).

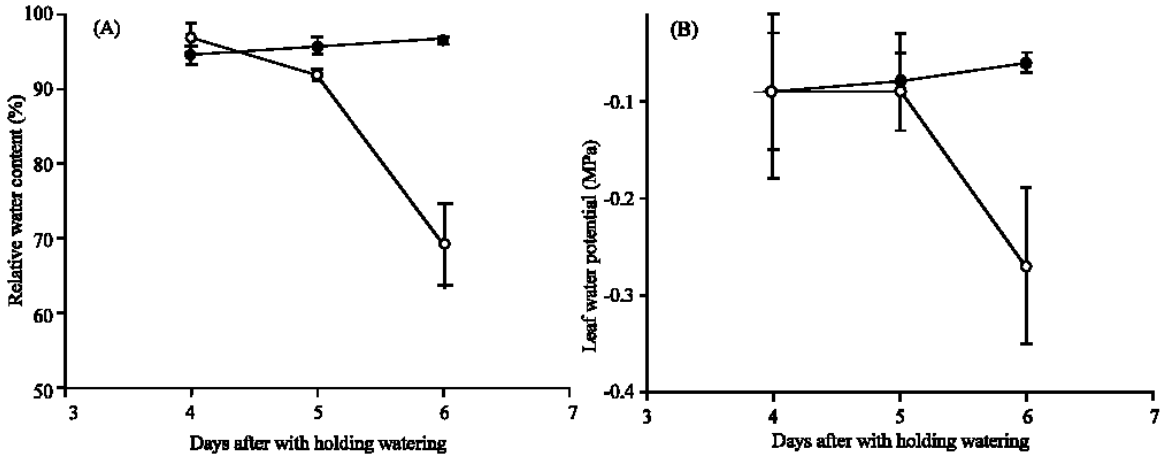

Fig. 1: Changes in relative water content (A) and leaf water potential (B) of peanut leaves variety Khon Kaen 4 during a period of water stress. Soil moisture contents in pots were maintained at field capacity (—●—) or under water deprivation (—○—). Vertical bars represent standard deviations

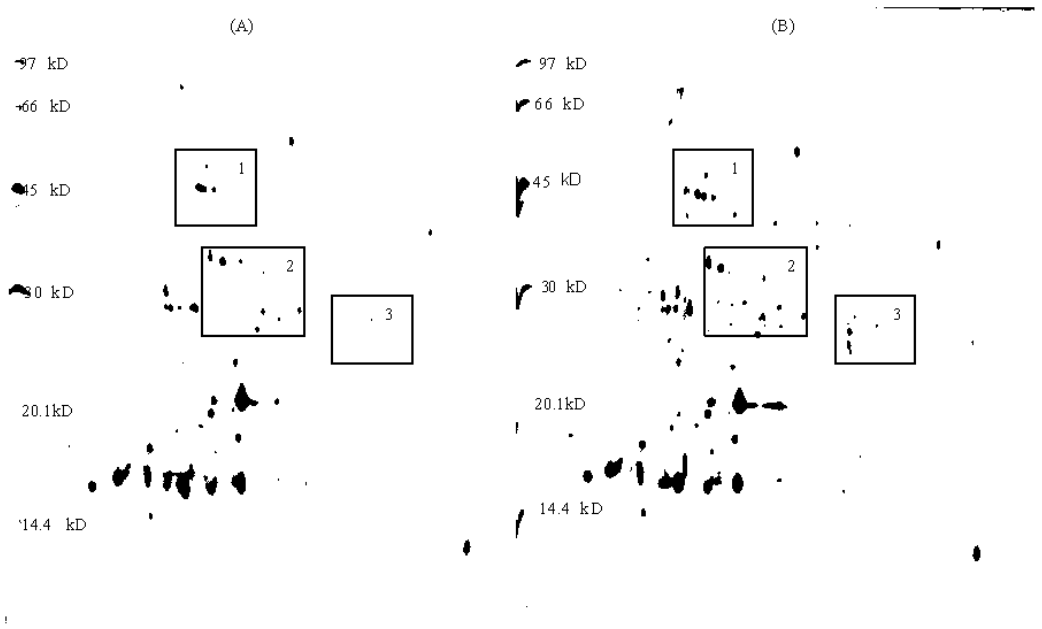

Fig. 2: The first replicate of proteomic map of peanut leaf proteins under 6 days of water deprivation (A) and adequate water supply (B). First dimensional focusing used 13 cm IPG strips with a linear pH gradient 4 to 7 loaded with 30 μg of total proteins for each strip. Proteins were separated in a second dimension using 12.5% SDS-PAGE. Proteins were visualized by silver nitrate staining. Molecular mass markers were on the left-hand column in each gel. Rectangular boxes encompass regions of differential protein expression

Differential expressions of proteins were detected. There were at least 6 proteins, protein no. 133, 190, 260, 277, 331 and 357, regulated by water status of peanut (Fig. 5). Protein No. 133 was in box 1, protein No. 190, 260 and 277 in box 2 and protein no. 331 and 357 in box 3 (Fig. 2 and 3). One protein, 190, was found in stressed plants only and referred to as inducible protein by water stress. The remaining five proteins, 133, 260, 277, 331 and 357 had greater percentage volume in non-stressed plants than in stressed plants. These proteins were referred to as down-regulated protein by water deficit.

Protein identities: Annotation for proteins using M_r and pI online resulted in at least six homologous proteins that could be putative proteins regulated by water deficit in peanut leaves (Table 1). The five proteins; 133, 190, 260,

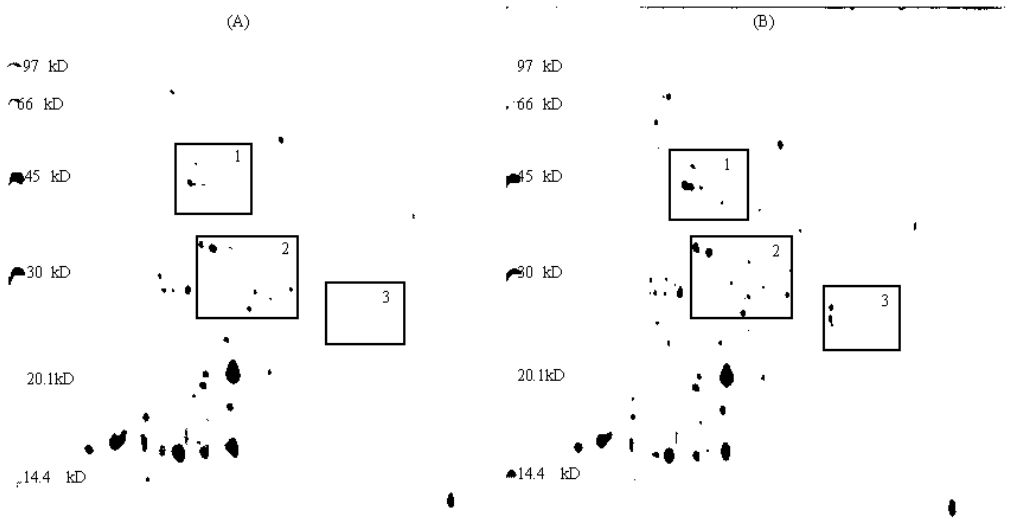

Fig. 3: The second replicate of proteomic map of peanut leaf proteins under 6 days of water deprivation (A) and adequate water supply (B). First dimensional focusing used 13 cm IPG strips with a linear pH gradient 4 to 7 loaded with 30 μg of total proteins for each strip. Proteins were separated in a second dimension using 12.5% SDS-PAGE. Proteins were visualized by silver nitrate staining. Molecular mass markers were on the left-hand column in each gel. Rectangular boxes encompass regions of differential protein expression

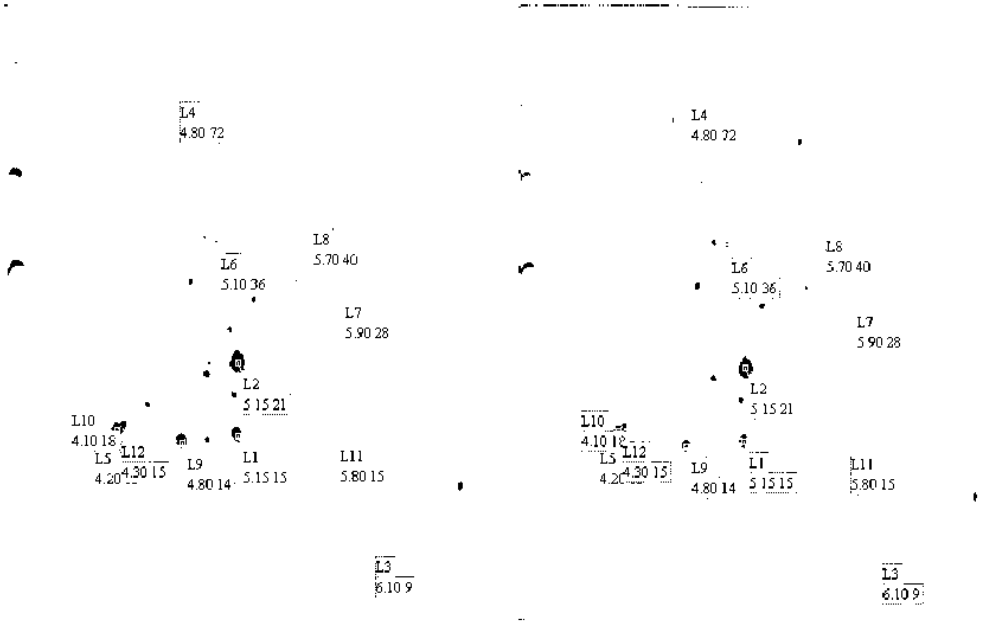

Fig. 4: An example of twelve reference spots of proteins in peanut leaf under 6 days water deprivation (A) and non-stressed plants (B). M_r and pI of reference spots (L1-L12) were calculated using protein molecular mass markers. These spots were used as reference for estimating M_r and pI of the remaining proteins

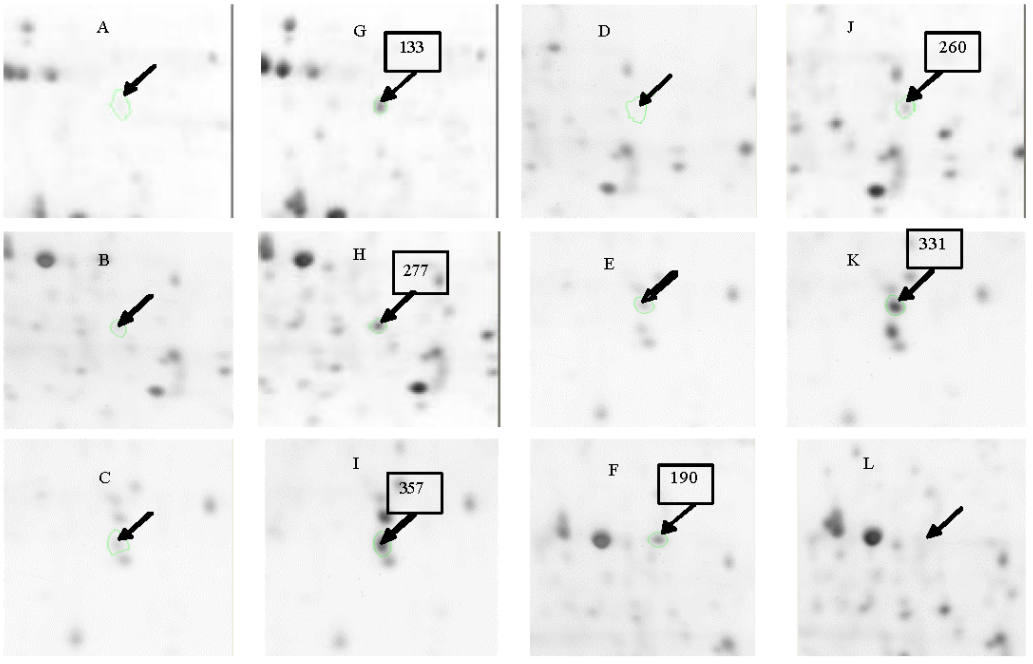

Fig. 5: Enlargement of selected regions in Fig. 2 and 3 to highlight some of differentially expressed protein spots. Proteins regulated by water status of peanut plants; A-F under 6 days of water deprivation, G-L under adequate water supply. Arrows in each region point to proteins that were regulated by water deficits. The spot number of a protein is in a rectangular box

Table 1: Properties of peanut proteins regulated by water stress and their protein homologous

		Protein homologous		
Protein No.	pI/Mr (kD)	Identity	Species/Protein Accession	pI/Mr (kD)
133	5.14/45	Chaperone protein DNAJ	*At*/Q8GYX8	5.17/44.7
190	5.14/36	Serine/threonine-protein phosphatase PP 1 isozyme	*At*/P48482	5.16/35.5
260	5.34/32	Peroxidase 43 precursor	*At*/Q9SZH2	5.36/32.7
277	5.18/31	SNF1-related protein kinase regulatory subunit beta-2	*At*/Q9SCY5	5.17/31.9
331	5.80/28	Auxin-responsive protein IAA29	*At*/Q93WC4	5.88/28.6
357	5.88/27	Caffeoyl-CoA O-methyltransferase	*Ze*/Q41720	5.88/27.6

277 and 33, were protein homologous in *Arabidopsis thaliana* (*At*) and only one protein, 357, was protein homolog in *Zinnia elegans* (*Ze*). M_r, pI and protein accessions were indicated. The homologous protein 190 was identified as serine/threonin-protein phosphatase PP 1 isozyme. The remaining homologous proteins were identified as chaperone protein DNAJ (133), peroxidase 43 precusor (260), SNF1-related protein kinase regulatory subunit beta-2(277), auxin-responsive protein IAA29 (331) and caffeoyl-CoA O-methyltransferase (357) (Table 1).

DISCUSSION

Withholding watering peanut variety Khon Kaen 4 for 6 days caused a large decrease leaf water potential and relative water content of the plants. Associated with a decrease in water status of peanut plants, protein expression was modulated. Among the water-stress-regulated proteins, two putative proteins are functional proteins including peroxidase 43 precursor, caffeoyl-CoA O-methyltransferase. Peroxidase 43 precursor is localized in cell wall and implicated in biosynthesis of lignin and cross-linking agents (Ralph *et al*., 2004). Caffeoyl-CoA O-methyltransferase is the enzyme responsible for synthesizing feruloylated polysaccharide in the pathway of lignin biosynthesis (Ye *et al*., 1994). Down regulation of these two proteins suggested the adverse effect of water stress on biosynthesis of components of cell walls and may affect the elasticity and/or integrity of cell wall. This evidence was previously reported in maize leaves under water stress (Vincent *et al*., 2005).

Chaperone protein DNAJ and auxin-responsive protein IAA29 are regulatory proteins. Chaperone protein DNAJ stimulates other molecular chaperones to maintain polypeptide in an unfolded, translocation-competent form (Zhou *et al*., 1995). IAA 29 is a key regulator of auxin-modulated gene expression (Remington *et al*., 2005). Water deficit appears to be critical to the processes regulated by these two proteins.

Serine/threonine-protein phosphatase PP1 and SNF1-related protein kinase regulatory subunit beta-2 are components of signal transduction. Serine/threonine-protein phosphatase PP1 is involved in signal transduction in eukaryotic organisms (Smith and Walker, 1996) and regulates actin organization and endocytosis (Chang *et al*., 2002), ion homeostasis in yeast (Williams-Hart *et al*., 2002) and K^+ channel in plant (Li *et al*., 1994). SNF1-related protein kinase regulatory subunit beta-2, a regulatory subunit of the SNF1-related protein kinase 2 (SnRK2) family in plants: (Hrabak *et al*., 2003). The role of the inducible protein, Serine/threonine-protein phosphatase PP 1, by water deficit in peanut plants could not be conclusive. However, the SNF1-related protein kinase 2 (SnRK2) is an osmotic-stress-activated protein kinase and a positive regulator of drought tolerance (Umezawa *et al*., 2004). Down regulation of this protein may be associated with an increase in sensitivity of the peanut variety to water deficit.

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