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Characterization of vegetative storage protein (VSP) and low molecular proteins induced by water deficit in stolon of white clover

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ABSTRACT

In stolon of white clover (*Trifolium repens* L.), the 17.3 kDa protein has been newly identified as a vegetative storage protein (VSP) which has preponderant roles in N accumulation and mobilization to sustain growth when capacity of N uptake is strongly reduced. To characterize the water deficit effect on this protein, the kinetic pattern of soluble protein, SDS–PAGE, Western blotting, and proteomic analysis was studied in the stolon of white clover during 28 days of water-deficit. Water deficit led to decrease protein concentration. SDS–PAGE revealed that two major proteins of 17.3 and 16 kDa were accumulated to high level in response to water stress. These proteins cross-reacted positively with antibodies raised against the 17.3 kDa VSP, a protein which shared biochemical features with stress proteins implied in dehydration tolerance. Using two-dimensional electrophoresis (2-DE) gel and matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) analysis, it was demonstrated that 19.5 and 17.3 kDa protein spots were up-regulated by water stress, and both spots were identical to nucleoside diphosphate kinase (NDPK) and lipid transfer proteins (LTPs), respectively. These results suggest that low molecular proteins induced by water-deficit in the stolon of white clover act as an alternative N reserves or play significant roles in plant protection against water-deficit stress.

1. Introduction

Water stress is one of the most important environmental factors inhibiting plant growth and development. Most plants have evolved mechanisms, followed by changes in key processes including protein synthesis, photosynthesis, respiration, and nucleic acid synthesis to cope with water deficit. As one of these processes, water stress causes the inhibition of *de novo* protein synthesis [1] as well as the induction of certain stress-specific proteins [2–5].

Among proteins that accumulated in plants in response to water stress, vegetative storage proteins (VSPs) which constitute the major nitrogenous storage pool in plants [6]. VSPs are simply known to associate not only with the regrowth potential after defoliation [7] but also with winter survival and spring growth [8,9]. For example, it has been found that VSP accumulate in alfalfa (*Medicago sativa* L.) during autumn and early winter, then rapidly decline at the early spring to provide a source of organic N for spring growth [8]. Rossato et al. [10] reported that VSP accumulation in the taproot of oilseed rape during flowering stages acts transient N storage buffer in response to N requirements during N filling of grain.

Several studies, stress-responsive plant proteins have been well documented in various species. Under drought stress conditions, various proteomic analysis has been reported in rapeseed [11], soybean [12] and alfalfa [13]. In rapeseed plants, 22 kDa protein (BnD22) was rapidly increased by water stress and involved in preservation of protein integrity [11]. Additionally, it has been also demonstrated that BnD22 which has high homology with water-soluble chlorophyll-binding protein leads plays a role in the protection of younger tissues from adverse condition by maintaining photosynthesis [14]. Similarly, three low molecular proteins of 21.8, 24.0 and 26.6 kDa were up-regulated in drought-stressed alfalfa leaves and identified as 2-cys peroxiredoxin-like protein, dehydroascorbate reductase and superoxide dismutase, respectively [13]. These proteins have been found to involve in

Abbreviations: 2-DE, two-dimensional electrophoresis; LTPs, lipid transfer proteins; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometer; NDPK, nucleoside diphosphate kinase; ROS, reactive oxygen species; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; VSP, Vegetative storage protein.

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detoxification of reactive oxygen species (ROS) [13,15]. Moreover, protein of 28 kDa accumulated in drought-stressed soybean root was identified as dehydrins, which has a function in abiotic stress tolerance by preservation of the structural integrity of the cell [12]. Drought stress-induced dehydrins accumulation and increase in its mRNA level have been reported in *Arabidopsis* [16,17] and in *Lathyrus sativus* [2]. These results suggested that low molecular proteins accumulation play as protective molecules against to drought stress.

To our knowledge, however, the specific proteins particularly VSP induced by water deficit stress has not yet been established in white clover. This work presents the VSP accumulation in the stolon of white clover exposed to prolonged water deficit and characterizes low molecular proteins having similar molecular weight with VSP by the proteomic analysis.

2. Materials and methods

2.1. Plant culture and experiment procedure

Sods of white clover at full vegetative stage were transplanted to 3 l pot containing a mixture of sand and fritted clay. Plants were regularly watered to field capacity during 2 weeks of adaptation. Water-deficit stress was imposed by decreasing the volume of water supply per day. 50 ml and 5 ml of daily irrigation per pot were applied to the well-watered (control) and water-deficit treatment, respectively, and half volume of the daily irrigation for each treatment was applied at 10:00 h and the other half at 16:00 h because considerable differences between Ψ_{soil} predawn and Ψ_{soil} midday were remarked when irrigated once per day [1]. Each treatment lasted for 28 days and stolon tissues were sampled at intervals of 7 days, respectively. Tissue samples were immediately frozen in liquid nitrogen. The freeze-dried samples were finely ground and stored under vacuum for further analysis.

2.2. Extraction and quantification of soluble protein

Proteins were extracted by suspending freeze-dried sample (200 mg) with 4 ml of 100 mM sodium phosphate buffer (pH 7.0) at 4 °C. Tubes were vortexed for 30 s 3 times and centrifuged at 13,000*g* at 4 °C for 10 min, and the supernatant was retained. The pellet was re-extracted. Soluble proteins in the combined supernatant were quantified using dye-binding method [18].

2.3. SDS–PAGE and Western blotting

For SDS–PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) analysis, 20 μg proteins were separated in 1.5 mm thick gels containing 12.5% acrylamide (propenamide) and stained with Coomassie Brilliant Blue R-250, or electroblotted onto a polyvinylidene difluoride membrane (Millipore, Saint Quentin en Yvelines, France) using a semi-dry blotting apparatus (Millipore). Low molecular mass markers were from BIORAD (Marne-La-Coquette, France). After the completion of protein transfer, blots were blocked overnight with TBST buffer (pH 8.0) containing 10 mM Tris-base, 150 mM NaCl and 0.15% (v/v) Tween-20. Antibodies were diluted in TBST buffer. The membranes were incubated for 90 min with polyclonal anti-17.3 kDa VSP antibodies diluted 1:5000 and for 120 min with goat anti-rabbit IgG alkaline phosphatase (BIORAD) diluted 1:5000 [19]. After washing in TBS buffer (pH 8.0) containing 10 mM Tris-base and 150 mM NaCl, blots were dipped briefly into alkaline phosphatase buffer pH 9.5 (0.1 M Tris–HCl, 0.1 M NaCl, 5 mM $MgCl_2$ $6H_2O$), and stained 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride prepared in alkaline phosphatase buffer as described by the manufacturer. VSP quantification was established by ELISA method as previously described by Noquet et al. [20].

2.4. 2-DE and MALDI-TOF-MS

2-DE was performed according to Lee et al. [21]. Briefly, each sample (150 μg of protein) was mixed in the sample buffer and then loaded onto IEF gel. SDS–PAGE in the second dimension was carried out as described by Laemmli [22]. The 2-DE gels were silver-stained according to Blum et al. [23].

Protein spots were excised from the gel, washed, in-gel reduced and S-alkylated followed by digestion with trypsin overnight at 37 °C. After overnight, peptide extracts and dry were performed according to Jensen et al. [24]. All samples were analyzed using a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA). Parent ion masses were measured in the reflectron/delayed extraction mode with an accelerating voltage of 20 kV, a grid voltage of 76%, a guide wire voltage of 0.01%, and a delay time of 150 ns. A two-point internal standard for calibration was used with des-Arg1-Bradykinin (m/z 904.4681) and neurotensin (m/z 1296.6853). Peptides were selected in the mass range of 500–3000 Da. For data processing, the software package PerSeptive-Grams was used. Database searches were performed using Protein Prospector (http://prospector.ucsf.edu). The following search parameters were applied: NCBI was used as the protein sequence database; a mass tolerance of 50 ppm and one incomplete cleavage were allowed; acetylation of the *N*-terminus, alkylation of cysteine by carbamidomethylation, oxidation of methionine, and pyroGlu formation of *N*-terminal Gln were considered as possible modifications.

2.5. Statistical analysis

A completely randomized design was used with three replicates for two water levels and five sampling dates. Duncan's multiple range test was employed to compare the means of separate replicates.

3. Results

3.1. Effect of water-deficit stress on protein concentration in stolon of white clover

In control plants, protein concentration increased during the first 14 days and then decreased to initial level, while it decreased for the first 7 days and then reached a plateau afterward in water deficit-stressed plants (Fig. 1). A significant difference observed between control and water deficit-stressed plants from 14 days after water-deficit treatment.

3.2. Protein pattern by SDS–PAGE and immunodetection of VSP in stolon of white clover

The variation in protein pattern in the stolon of control and water deficit-stressed plants was assessed by SDS–PAGE (Fig. 2). Two polypeptides with relative molecular masses of 17.3 and 16 kDa started to accumulate at 14 days of water-deficit stress and remained at a higher level until 28 days. In control plants, the protein profile was not significantly changed throughout experimental period. To identify putative vegetative storage proteins (17.3 and 16 kDa), we used Western blot analysis (Fig. 3A). It revealed that the polyclonal antibodies were raised against the 17.3 kDa VSP of white clover [19] cross-reacted with both 17.3 and 16 kDa polypeptides. Compared to well-watered plants, the

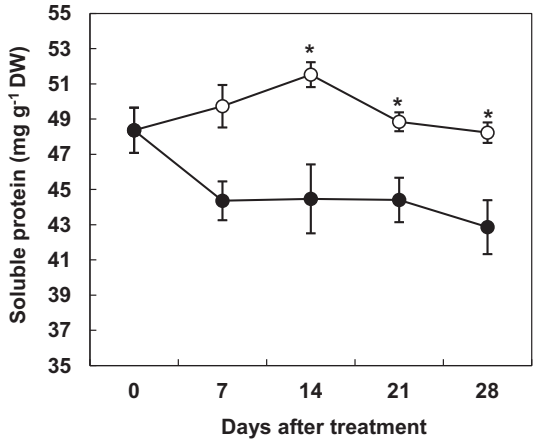

Fig. 1. Changes in soluble protein concentration in stolon of white clover during 28 days under well-watered (○) or water-deficient (●) condition. Each value is the mean ± standard error $n = 3$. Asterisks indicate control plants values that are significantly different from those of water-deficient plants at $P \leqslant 0.05$.

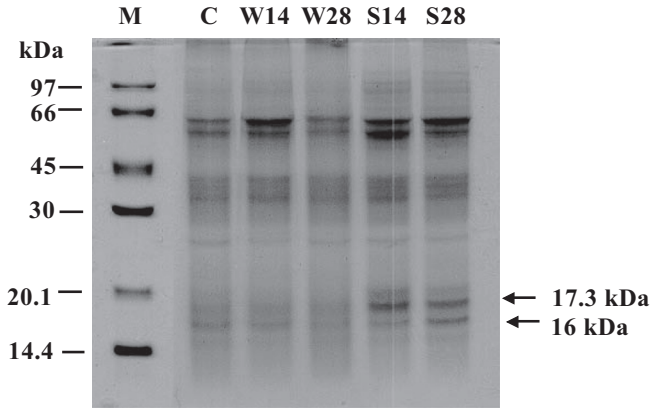

Fig. 2. SDS–PAGE analysis of soluble proteins extracted from stolon of well-watered or water-deficit plants. Tissue samples were taken at the day of treatment (control, day 0), 14 and 28 days after well-watered (W) or water-deficit (S) treatment. A constant amount of 20 μg proteins was loaded in each well. Molecular mass markers (kDa) are listed on the left of the gel.

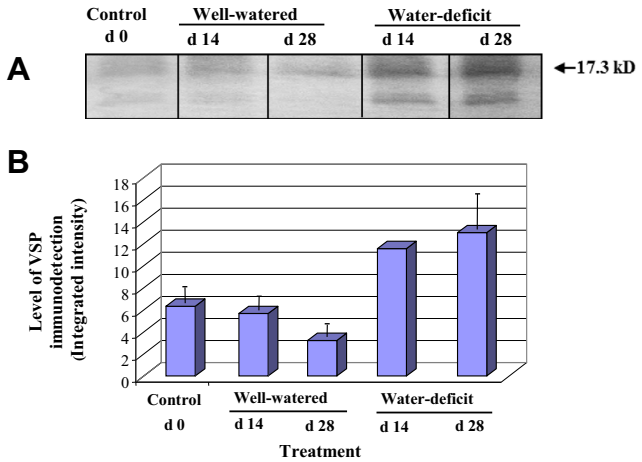

Fig. 3. Changes in Western blotting analysis of 17.3 kDa VSP by immunodetection with anti 17.3 kDa VSP antibodies (A) and level of VSP immunodetection (B). Tissue samples were taken at the day of treatment (control, day 0), 14 and 28 days after well-watered or water-deficit treatment.

level of VSP was 2-fold (after 14 days) and 4-fold (after 28 days) in water stressed-plants (Fig. 3B).

3.3. 2-DE analysis and identification of proteins by MALDI-TOF-MS

The silver-stained 2-DE protein patterns showed overall clear differences in the spots between control and water deficit-stressed plants (Fig. 4). Twenty-one proteins were induced by water-deficit treatment but we focused our attention on the low molecular weight spots within a range of 15–19 kDa, in which identified VSP involve. As shown by boxes, three spots detectable in the water-stressed stolon were present at high levels (marked 1, 2, 3 arrows). Its molecular was 19.5 and 17.3 kDa, respectively, and it is similar to that of band of 17.3 kDa observed in on the gel. Identification by MALDI-TOF-MS revealed that polypeptide of 19.5 kDa with pI 5.2 (spot No. 1) was identical to nucleoside diphosphate kinase (NDPK) in *Microbulbifer* (Table 1). Spot number 2 at 17.3 kDa with pI 4.9 matched with putative lipid transfer protein in *Oryza sativa* (Table 1). Spot number 3 protein is not matched any other proteins.

4. Discussion

The water deficit treatment resulted in the gradual decrease from −0.46 to −2.33 MPa in leaf water potential (Ψ_w) in water-stressed plants, while no significant changes occurred in control plants (data not shown). Protein concentration of stolon in water deficit-stressed plants was always significantly lower than well-watered (control plants) (Fig. 1). Using the SDS–PAGE analysis, we found that two major polypeptides of 17.3 and 16 kDa were accumulated to high level in response to exposure to water deficit (Fig. 2). In leaves and roots, the protein profile was not significantly changed in both treatments throughout experimental period (data not shown). It was noteworthy that there were significant changes in protein profile in the stolon, which has been known to be the site of N reserve storage [25]. Therefore, it can be suggested that two polypeptides (17.3 and 16 kDa) found in stolon have the VSP characteristics, which exhibit a catalytic or physiological function in addition to their role in nitrogen storage [19,25]. These two prominent polypeptides were cross-reacted with the anti-VSP antibodies of white clover indicating that the 17.3 and 16 kDa proteins are VSP. It could be also assumed that the 16 kDa protein is an immature or a degraded form of the 17.3 kDa VSP. Western blots also showed that as water stress prolonged, that accumulation of VSPs increased markedly (Fig. 3). VSP accumulation by water deficit was also observed in white clover during low temperature stress in nodules [19]. These results suggest that VSP might be involved in the transient storage of N that occurred during N recycling processes provoked under conditions of numerous abiotic and biotic constraints [9,26], especially dehydration stress. Indeed, this VSP accumulation may allow plants not only to give stress tolerance but also to subsequently grow effectively by sequestering the endogenous N protein form until more favorable conditions for growth arrive. Close [27] reported the presence of dehydrins ranging from 15 to 120 kDa in several species of monocotyledons, dicotyledons and of few gymnosperms, and suggested that these proteins play a protective role during environmental stresses leading to plant dehydration such as water deficit or low temperature.

The white clover cDNA clone (GenBank accession No. AY059460) encoding the 17.3 kDa VSP possesses high homology with pathogenesis related proteins (PRP), anti-freeze proteins (AFP) and abscisic acid (ABA)-responsive proteins of several legume species [19]. VSP of white clover exhibit common biochem-

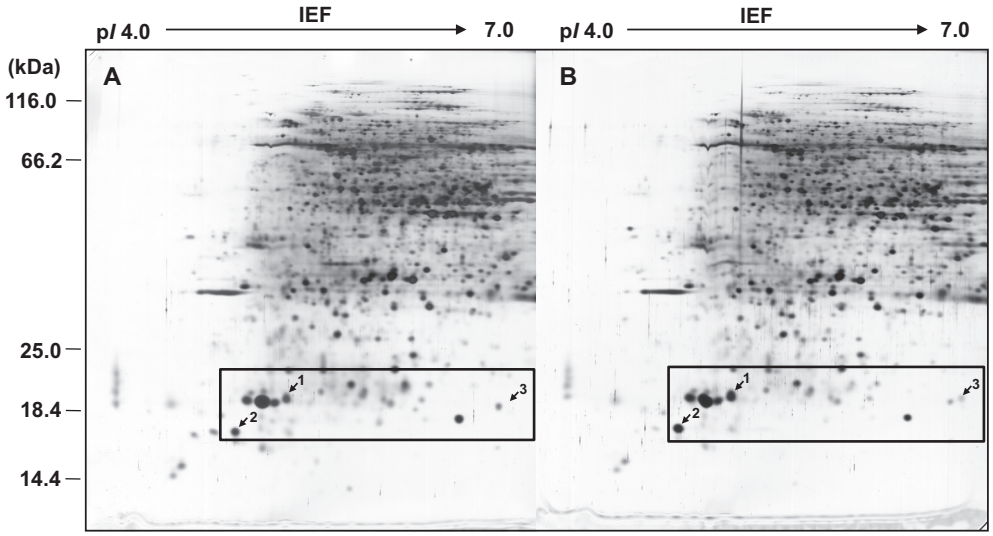

Fig. 4. 2-DE analysis of total proteins induced by water-deficit treatment at 28 days. Protein sample (150 μg) were separated on 2D-gel (pI 4–7), and silver-stained. Representative 2-DE gel images of a control (A) and water-deficit plants (B). The arrows indicate proteins induced or reduced over control. The relative Mr are indicated on the left side in kDa. Data analysis was performed using PDQuest software program.

Table 1
Identification of spots highly induced in white clover stolon after 28 days of water-deficit treatment.

Spot No./change	Protein name	Acc. No.[a]	Species	Theoretical/ observed pI	Theoretical/observed Mr (kDa)	PM[b]	SC (%)[c]
1/Up	COG0105: nucleoside diphosphate kinase	ZP_00316699	*Microbulbifer degradans 2–40*	5.3/5.2	15.4/19.5	7	39
2/Up	Putative lipid transfer protein	NP_922652	*Oryza sativa*	9.1/4.9	16.0/17.3	9	25
3/Up	ND[d]			/6.8	/19.5		

[a] Accession number from NCBI database.
[b] Number of peptides matched.
[c] Sequence coverage by peptide mass fingerprinting using MALDI-TOF-MS.
[d] Not good matched.

ical features of stress responsive proteins which have high content of neutral amino acids that are conducive to the formation of alpha helices and heat stability typical of hydrophilic proteins [19]. VSP accumulation induced by external ABA application and their corresponding transcripts has been also reported [9,19].

In this study, two proteins with molecular mass of 19.5 kDa (spot No. 1) and 17.3 kDa (spot No. 2) differentially expressed by prolonged water deficit for 28 days were found in the stolon of white clover, and both spots were up-regulated (Table 1). MALDI-TOF-MS analysis revealed that two polypeptides of 19.5 kDa (spot No. 1) and 17.3 kDa (spot No. 2) were homologous to nucleoside diphosphate kinase (NDPK) and putative lipid transfer proteins (LTPs), respectively (Table 1). Nucleoside diphosphate kinase (NDPK) has well-known to have function in the general homeostasis of cellular nucleoside triphosphate pools [28,29]. NDPK has also multiple functions, participating in phytochrome A response [30], UV-B signalling [31], cold stress tolerance [28] and nitrogen mobilization [32]. In addition, it has been recently reported that NDPK is associated with signal transduction pathways involved in oxidative stress [29]. In transgenic plants overexpressing NDPK2, high levels of NDPK activity has been found to be associated with the increased ascorbate peroxidase (APX) activity [29], and resulted in a lower levels of ROS than wild types as a protective regulator in the down-regulation of the cellular redox state [28]. Lipid transfer-proteins (LTPs) are small, basic and secreted proteins [33]. It has been suggested that LTPs involve in resistance of bacterial pathogen [34], mediate cell-wall loosening *in vitro* [35], and export of lipids to the plant surface [36]. Recently, induction of LTP3 has been observed under freezing and drought stress, and its overexpression results in constitutively enhanced stress tolerance [37,38], suggesting that LTP3 might act as a co-signal of lipids and bind and transfer these lipids from cytosol to the cell membrane or cell wall to form cuticular wax. Accordingly, accumulation of these low molecular proteins might mediate water stress tolerance by signalling the activation of antioxidant enzymes to scavenge of ROS and/or prevent loss of water.

Taken together, our results suggest that VSP of white clover stolon induced by water-deficit acts as an alternative N reserve, and low molecular proteins having similar molecular weight with VSP play a significant role in plant protection against water deficit stress. Further work will be examined whether the accumulation of VSP during water-deficit stress can improve the recovery of growth when water condition becomes more favorable.

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