Biochemical and Biophysical Research Communications 443 (2014) 229–233

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Bok-Rye Lee [a,b], Dong-Gi Lee [c], Jean-Christophe Avice [d], Tae-Hwan Kim [a,*]

[a] Department of Animal Science, Institute of Agricultural Science and Technology, College of Agriculture and Life Science, Chonnam National University, P.O. Box 205, Buk-Gwangju, South Korea
[b] Bioenergy Research Center, Chonnam National University, Gwangju 500-757, South Korea
[c] Division of Life Science, Korea Basic Science Institute, Daejeon 305-806, South Korea
[d] INRA, UMR INRA/UCBN 950 Ecophysiologie Végétale, Agronomie et Nutritions NCS, IFR 146 ICORE, Institut de Biologie Fondamentale et Appliquée, Université de Caen Basse-Normandie, F-14032 Caen, France

ARTICLE INFO

Article history:
Received 13 November 2013
Available online 2 December 2013

Keywords:
Proteomics
Vegetative storage protein (VSP)
Water-deficit
White clover

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.

* Corresponding author. Fax: +82 62 530 2129.
E-mail address: grassl@chonnam.ac.kr (T.-H. Kim).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.11.087

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