Journal of Plant Physiology 170 (2013) 828–837

Contents lists available at SciVerse ScienceDirect

Journal of Plant Physiology

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

Physiology

Cold stress causes rapid but differential changes in properties of plasma membrane H^+-ATPase of camelina and rapeseed

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ARTICLE INFO

Article history:
Received 27 August 2012
Received in revised form 5 January 2013
Accepted 10 January 2013
Available online 8 February 2013

Keywords:
Acclimation
Brassica napus
Cold stress
Camelina sativa
PM H^+-ATPAse

ABSTRACT

Camelina (*Camelina sativa*) and rapeseed (*Brassica napus*) are well-established oil-seed crops with great promise also for biofuels. Both are cold-tolerant, and camelina is regarded to be especially appropriate for production on marginal lands. We examined physiological and biochemical alterations in both species during cold stress treatment for 3 days and subsequent recovery at the temperature of 25 °C for 0, 0.25, 0.5, 1, 2, 6, and 24 h, with particular emphasis on the post-translational regulation of the plasma membrane (PM) H^+-ATPase (EC3.6.3.14). The activity and translation of the PM H^+-ATPase, as well as 14-3-3 proteins, increased after 3 days of cold stress in both species but recovery under normal conditions proceeded differently. The increase in H^+-ATPase activity was the most dramatic in camelina roots after recovery for 2 h at 25 °C, followed by decay to background levels within 24 h. In rapeseed, the change in H^+-ATPase activity during the recovery period was less pronounced. Furthermore, H^+-pumping increased in both species after 15 min recovery, but to twice the level in camelina roots compared to rapeseed. Protein gel blot analysis with phospho-threonine anti-bodies showed that an increase in phosphorylation levels paralleled the increase in H^+-transport rate. Thus our results suggest that cold stress and recovery in camelina and rapeseed are associated with PM H^+-fluxes that may be regulated by specific translational and post-translational modifications.

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Introduction

The need to advance bio-fuels as renewable energy sources is ever-increasing due to concerns over global warming and the fluctuation of oil prices. Studies to improve the efficiency of bio-energy production have focused on available bio-resources (Sticklen, 2008), adjustment of oil composition in crops (Pinzi et al., 2011), oil refining processes (Santori et al., 2012) and development of species which are resistant to various environmental stress conditions (Baltruschat et al., 2008; Huang et al., 2009; Santiago et al., 2009; Chen and Murata, 2011; Inostroza-Blancheteau et al., 2012).

The most important factors limiting bio-energy production from plant resources are optimal crop management systems and stress tolerance or resistance in biofuel crops. The correct selection of bio-energy crops depends on the climatic characteristics, especially the temperature range, and specific soil conditions in the region. The present study involved two bio-energy crops that have potential for bio-fuel production. Camelina (*Camelina sativa*) is a rising bio-energy crop with potential for low-input, low cost oil production under a relatively wide range of different climate and soil conditions (Budin et al., 1995). Furthermore, its short life cycle (about 90 days) is an advantage for multi-cropping or cultivation in regions with short growing seasons. Rapeseed (*Brassica napus*) is a cold tolerant species that is already a popular crop for bio-diesel production. Rapeseed oil is enriched in oleic acid (C18:1), which is suitable for bio-diesel fuel for winter seasons because of its high boiling point (Budin et al., 1995). Camelina oil also contains a high amount of unsaturated fatty acid, making it suitable for future use as a replacement for petroleum (Zubr, 1997).

During the winter in temperate regions, crops are exposed to cold stress as the temperature nears and drops below 0 °C. Cold stress can cause micro-organelle disruption, phase transition in cell membrane lipids, ionic imbalance in the cytosol, as well as the inhibition of crop growth and development, which consequently reduces crop yield and production (Lyons and Raison, 1970). While freezing is lethal to most crops, winter crops can survive by maintaining a basal metabolism at low temperatures (Weiser, 1970; Liu

Abbreviations: BSA, bovine serum albumin; BTP, 1,3-bis(tris[hydroxymethyl] methylamino) propane; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EDTA, ethylene diamine tetraacetic acid; MOPs, 3-[N-molpholino] propanesulfonic acid; PBST, phosphate buffered saline-Tween 20; PMSF, phenyl methane sulfonyl fluoride; PVP, polyvinylpyrrolidine; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

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http://dx.doi.org/10.1016/j.jplph.2013.01.007

et al., 2002). To produce bio-fuel crops on marginal land in the winter season, knowledge of physiological responses of the crops is necessary to prevent abiotic stress-related productivity losses. The discovery of novel mechanisms or responses under cold stress will provide new opportunities to improve stress resistance within crop species.

In previous studies, we have reported that differences in biochemical responses to cold stress between cold-tolerant and -sensitive plant species are associated with changes in plasma membrane (PM) H^+-ATPase enzymes (Ahn et al., 1999, 2000). In addition, it has been previously reported that modulation of PM H^+-ATPase activity, and 14-3-3 protein translation, and phosphorylation of enzymes occurs in the cold-sensitive species cucumber during and after cold stress (Janicka-Russak et al., 2012). Also, other abiotic stress agents, such as excess metals (Ahn et al., 2001; Shen et al., 2005; Ahn and Matsumoto, 2006; Janicka-Russak et al., 2008), salt (Yamashita and Matsumoto, 1997; Pitann et al., 2009; Sahu and Shaw, 2009; Wakeel et al., 2010, 2011), mechanical injury (Oufattole et al., 2000) and drought (Gong et al., 2010) can affect the activity of PM H^+-ATPase, which is regulated post-translationally (Gaxiola et al., 2007). PM H^+-ATPase proteins are encoded by a gene family of 11 members in *Arabidopsis* that are tightly regulated transcriptionally by a variety of physiological responses (Palmgren, 2001). PM H^+-ATPase is a master enzyme that can generate an electrochemical gradient for secondary active transport through the phosphorylation of a C-terminal autoinhibitory domain (Fuglsang et al., 1999). The enzyme activity of PM H^+-ATPase can be harnessed to drive the outside-to-inside transport across the PM of a variety of solutes and ions, including K^+, NO_3^-, nucleotides or nucleosides, peptide, amino acids, hexoses and sucroses (Palmgren, 2001).

The 14-3-3 proteins are involved in the auto-phosphorylation of PM H^+-ATPase (Fullone et al., 1998). The 14-3-3 proteins can bind if a serine or a threonine amino acid residue is phosphorylated in the 14-3-3 protein's binding site in the target protein (Fuglsang et al., 1999). Rosenquist et al. (2000) reported that the twelve 14-3-3 protein family isoforms in *Arabidopsis* have different binding affinities. Regulation of 14-3-3 binding to PM H^+-ATPase, and suppression of 14-3-3 binding by the *Arabidopsis* chaperone J3, through interaction with PKS5 kinase, was reported in recent studies (Duby et al., 2009; Ekberg et al., 2010; Hayashi et al., 2010).

In this study, we characterized the effects of cold stress and recovery from cold stress on the physiological and biochemical properties of PM H^+-ATPase, which may be critical in cold tolerance mechanisms in camelina and rapeseed. To ascertain the physiological changes which take place at the initiation of cold treatment and during recovery in the two crops, electrolyte leakage, chlorophyll fluorescence and stomatal conductance were measured. Furthermore, the relationships between protein translation and activity and post-translational regulation by threonine-phosphorylation of PM H^+-ATPase were analyzed in PM samples isolated from leaves and roots of cold-treated plants and plants at each stage of recovery from stress of both species.

Materials and methods

Plant material

Camelina (*Camelina sativa* L. cv. Crantz) and rapeseed (*Brassica napus* L. cv. Youngsan) seeds were sown in a 5-L hydroponic culture tray on nylon mesh or rock wool in half-strength Hoagland solution and germinated at 25 °C in the dark for 3 days. About 300 seeds of each were sown on nylon mesh, or 10 seeds of each sown on rock wool. After germination, seedlings were grown under a 16 h light/8 h dark cycle (180 $\mu mol\ m^{-2}\ s^{-1}$). The relative humidity was 70% and nutrient solutions were changed every 3 days. To determine the critical temperature of cold stress, 7-day-old seedlings were moved to a HB-303-DH-L cold chamber (Hanbaek Science, Korea) operating at 16, 8, or 2 °C for 3 days to test for electrolyte leakage. To investigate physiological changes, 3-week-old plants grown on the hydroponic system were moved to the same cold chamber operating at 2 °C for 1, 3, and 5 days. Plants treated at 2 °C for 3 days were returned directly to room temperature (25 °C) for recovery, including replacement of the hydroponic nutrient solution with solution at room temperature. These plants were used for plasma membrane (PM) isolation during recovery at 25 °C.

Electrolyte leakage test

Electrolyte leakage was measured following the method of Yu et al. (2006). Leaves (0.2 g) of each plant were cut into 5 mm slices and put in a test tube containing 30 mL of deionized water. Samples were shaken at 100 rpm at 26 °C for 2 h, and the electrical conductivity of the solution (EC1) was measured using an IQ170 electrical conductivity meter (IQ scientific instruments, USA). Autoclaved samples (EC2) were measured to determine the maximum percentage of electrolyte leakage. Relative electrical conductivity (REC) was calculated as:

$$\mathrm{REC}\ \ (\%) = \frac{\mathrm{EC1}}{\mathrm{EC2}} \times 100.$$

Chlorophyll fluorescence and stomatal conductance measurement

Chlorophyll fluorescence and stomatal conductance were measured according to the procedure of Zhang et al. (2005) and Frei et al. (2008), and measurements were carried out on 3-week-old plants in wound-free third main leaves with an OS1-FL fluorescence meter (Opti Science, USA) and model SC-1 leaf porometer (Decagon Devices, USA), respectively. Three-week-old plants were used because the instruments required at least 10 mm diameter of leaf surface. Before measurement of chlorophyll fluorescence, leaf samples were pretreated in a dark chamber clip for 30 min. Stomatal conductance was measured on the abaxial side of the leaf after 3 h light period, which is the time point known to provide the maximum stomatal opening (Shimazaki et al., 2007).

Isolation of PM vesicles

PM vesicles were prepared at 4 °C as previously described (Palmgren et al., 1990). In brief, leaf and root samples were collected from control or temperature-treated plants. Samples were ground in ice-cold homogenization buffer containing 50 mM MOPs–BTP (pH 7.5), 330 mM sucrose, 5 mM EDTA, 5 mM dithiothreitol (DTT), 0.5 mM phenylmethanesulphonylfluoride, 0.2% (w/v) casein, 0.2% bovine serum albumin, and 0.5% PVP-40. The homogenate was filtered through four layers of cheesecloth and the filtrate was centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatant was collected and centrifuged at 80,000 × *g* for 45 min, and the resulting precipitate was resuspended in buffer consisting of 330 mM sucrose, 5 mM potassium phosphate (KH_2PO_4; pH 7.8), 5 mM KCl, 0.1 mM EDTA, and 1 mM DTT. The homogenate was loaded onto a 12 g two-phase system containing 6.5% Dextran T-500 (Sigma–Aldrich, USA), 6.5% (w/w) polyethylene glycol (PEG)-3350 (Sigma–Aldrich), 250 mM sucrose, 5 mM KH_2PO_4 (pH 7.8), 4 mM KCl, and sterile DW. After the batch procedure, the resulting upper phase was mixed with a dilution buffer consisting of 5 mM MOPs–BTP (pH 7.5), 330 mM sucrose, and 5 mM KCl, and was centrifuged at 100,000 × *g* for 60 min. The carefully obtained PM vesicles were either used immediately or stored at −80 °C until

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