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A vacuolar H^+-pyrophosphatase differential activation and energy coupling integrate the responses of weeds and crops to drought stress ☆

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ABSTRACT

Background: *Cyperus rotundus* L. is a C4 weed of large vegetative and reproductive vigor endowed with competitive advantages over most crop species mainly under adverse environmental conditions. Vacuole functions are critical for the mechanisms of drought resistance, and here the modulation of the primary system of vacuolar ion transport is investigated during a transient water stress imposed to this weed and to C4 crop species (*Zea mays* L.).
Methods: The vacuolar H^+ pumps, the H^+-ATPase and H^+-PP_iase, expression, activities and the energy coupling were spectrophotometrically investigated as key elements in the differential drought-resistance mechanisms developed by weeds and crops.
Results: In *C. rotundus* tonoplasts, ATP hydrolysis was more sensitive to drought than its coupled H^+ transport, which was in turn at least 3-folds faster than that mediated by the H^+-PP_iase. Its PP_i hydrolysis was only slightly affected by severe water deficit, contrasting with the disruption induced in the PP_i-dependent H^+-gradient. This effect was antagonized by plant rehydration as the H^+-PP_iase activity was highly stimulated, reassuming a coupled PP_i-driven H^+ pumping. Maize tonoplasts exhibited 2–4 times lower hydrolytic activities than that of *C. rotundus*, but were able to overactivate specifically PP_i-dependent H^+ pumping in response to stress relief, resulting in an enhanced H^+-pumps coupling efficiency.
Conclusion: These results together with immunoanalysis revealed profiles consistent with pre- and post-translational changes occurring on the tonoplast H^+-pumps, which differ between weeds and crops upon water deficit.
General significance: The evidences highlight an unusual modulation of the H^+-PP_iase energy coupling as a key biochemical change related to environmental stresses adaptive capacity of plants.

1. Introduction

Purple nutsedge (*Cyperus rotundus* L.) has been considered by many authors the world's worst weed. A befitting designation for a species spread throughout in more countries (at least 92) than any other weed, and which infests at least 52 different crops worldwide [1]. It grows in all types of soils and can survive the highest temperatures known in agriculture [2]. While it prefers moist soil, established nutsedge plants will thrive even under severe dry conditions. From the physiological point of view, these characteristics have been correlated with its high C4 metabolism efficiency, usually associated with the more invasive weeds. Indeed, C4 weeds have clear competitive advantage over C3 crop species under many field situations, especially in dry situations where stomatal conductance is low [3], and under high light intensities and temperatures [4]. Moreover, while weed and crop species differing in photosynthetic pathway (C_4 vs C_3) are likely to respond differently to these factors, it is not clear at what extent weeds and crops sharing the same photosynthetic metabolism (C_4 vs C_4) can differ in their stress tolerance-mechanisms [5]. Actually, nutsedge plants can also suppress other C_4 species growing in its vicinity, and efforts to establish the biochemical bases of such success

Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; DTT, dl-dithiothreitol; EDTA, ethylenediamine tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; PVP-40, polyvinylpyrrolidone-40

☆ *In memoriam*: Prof. Claudio A. Retamal, a deeply kind and generous colleague and an unforgettable friend.

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in dominating natural and agricultural habitats have mainly been focused on its ability to produce allelopathic substances [6].

On the other hand, stressful levels of environmental factors such as water and nutrient availability influence weed/crop interactions directly and also may interfere with weed control [5]. Weeds are highly efficient in water and nutrient acquisition, and thus are very competitive affecting even the most efficient crops developed for the agriculture. Drought is one of the most important environmental stresses limiting the productivity of crops around the world, and the comprehension of the different drought tolerance mechanisms is very important to develop new strategies to cope with this problem [7,8].

Drought-tolerant plants maintain their turgor at low water potentials by increasing solute molecules in the cell [9], a phenomenon tightly regulated in order to maintain the cell homeostasis. Indeed, in many cell types, any perturbations of the osmotic balance rapidly activate mechanisms involved in cell volume recovery, and the first responses involve changes in ion fluxes across the plasma membrane and vacuoles, which rapidly result in water flow across the cell membranes and induce changes in cell volume, and vice versa [10,11]. The vacuolar H^+-ATPase (V-ATPase; EC 3.6.1.3) plays important roles in controlling the cytoplasmic pH and ion fluxes in plant cell, which are essential for cellular processes including plant adaptation to stressful growth conditions [12]. However, a second proton pump, a vacuolar H^+-pyrophosphatase (V-PP_iase; EC 3.6.1.1), coexists with V-ATPase in the tonoplast (vacuolar membrane). Several evidences indicate that these enzyme functions like a backup to V-ATPase at limited energetic conditions [e.g., 13,14]. Furthermore, transgenic overexpression of the gene encoding the V-PP_iase improves drought and salt tolerance in different species, for example, in *Arabidopsis* [15], tobacco [16], maize [17] and cotton [18,19].

In order to provide new insights on the physiological and biochemical roles of the tonoplast H^+ pumps in the development of different drought tolerance mechanisms, we compared the capacities of one of the most invasive weeds (*C. rotundus* L.) and a globally important C_4 crop species (*Zea mays* L.) to cope with water deficit. A differential activation and energy coupling were found for the V-PP_iase, which seems to be related to the differential adaptive responses of weeds and crops to drought stress at the same time that it provides further support to recent findings that have motivated a reconsideration of the current knowledge of the role of V-PP_iase in plant physiology [20,21].

2. Materials and methods

2.1. Plant material

Cyperus rotundus L. and *Zea mays* L. (variety UENF 506-6) were cultivated in greenhouse, in pots with a substrate mixture of 65% sand and 35% commercial soil. Cultivated (15-days-old) plants were irrigated daily and 20 days after germination drought was imposed by withholding irrigation for about 11 days, plant substrate was monitored for its water content, and samples processed for analysis when the substrate water reached 30 and 70% of deficit, and also 24 h after re-watering. The substrate water content was measured by the Gravimetric Technique, in which a soil sample is collected, accurately weighted, completely dried out in an oven, re-weighted and the soil moisture percentage is calculated from the weight loss. Ten pots were maintained upon uninterrupted irrigation as controls.

2.2. Preparation of tonoplast-enriched membrane vesicles

Tonoplast vesicles were isolated from *C. rotundus* pseudostems or maize mesocotyls using differential centrifugation essentially as described by Giannini and Briskin [22], with minor modifications. About 50 g of mesocotyl was homogenized using a mortar and pestle in 2 mL/g (fresh weight) of ice-cold buffer containing 10% (v/v) glycerol, 0.5% (v/v) PVP (PVP-40, 40 kD), 5 mM EDTA, 0.13% (w/v) BSA, and 0.1 M Tris–HCl buffer, pH 7.6. Just prior to use, 150 mM KCl, 3.3 mM DTT, and 1 mM PMSF were added to the buffer. The homogenate was strained through four layers of cheesecloth and centrifuged at 10,000 ×*g* for 10 min. The supernatant was centrifuged once more at 10,000 ×*g* for 10 min and then at 100,000 ×*g* for 30 min. The pellet was resuspended in a small volume of ice-cold buffer containing 10 mM Tris–HCl, pH 7.6, 10% (v/v) glycerol, 1 mM DTT, and 1 mM EDTA. The suspension containing the mesocotyl vesicles was layered over a 25/46% (w/w) discontinuous sucrose gradient that contained, in addition to sucrose, 10 mM Tris–HCl buffer, pH 7.6, 1 mM DTT, and 1 mM EDTA. After centrifugation at 100,000 ×*g* for 2 h in a swinging bucket, the membrane vesicles were collected at the interface between 25 or 46% sucrose. All preparative steps were performed at 4 °C. The vesicles were frozen under liquid N_2 and stored at −70 °C until use. Protein concentrations were determined by the Bradford's method [23].

2.3. Substrate hydrolysis assay

ATPase and PP_iase activity was determined colorimetrically by measuring the release of P_i as described by Fiske and Subbarow [24]. The reaction media contained 50 mM Tris–HCl, pH 6.5 or 7.0, 3 mM $MgSO_4$, 100 mM KCl, 1 mM ATP or 0.1 mM PP_i. The reaction was started by addition of tonoplast protein (40 μg/mL) and stopped with ice-cold 5% (w/v) trichloroacetic acid after 30 min of incubation at 25 °C. The H^+-ATPase activity was measured with and without 100 mM KNO_3 or 10 nM Bafilomycin and the nitrate/Bafilomycin-sensitive activity was attributed to the V-ATPase [25]. The PP_iase activity stimulated by 100 mM KCl was considered as the V-PP_iase [26]. V-PP_iase activity was calculated as half of the rate of P_i released from PP_i (μmoles PP_i hydrolyzed per minute).

2.4. Proton pumping assay

ATP- and PP_i-dependent H^+ transport across membranes was measured as the initial rate of fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) at 25 °C using a fluorimeter (model F-3010, Hitachi, Tokyo) using a protocol adapted by Facanha and de Meis [27]. The excitation wavelength was set at 415 nm and the emission wavelength was set at 485 nm. The reaction medium contained 10 mM Tris–HCl, pH 7.0, 2 μM ACMA, 5 mM $MgCl_2$, 100 mM KCl, 1 mM ATP or 0.1 mM PP_i. The reaction was initiated by the addition of 50 μg/mL of membrane vesicles. The addition of 20 mM NH_4Cl was used to show a recovery of the fluorescence that indicated a collapse of the preliminarily formed H^+ gradient.

2.5. Western blot assays

Tonoplasts were isolated from *C. rotundus* pseudostems or maize mesocotyls. A sample of membrane proteins (30 μg), separated in a 12% SDS-PAGE was immunoblotted with antibodies against the subunit B of V-H^+-ATPase or against H^+-PP_iase (Agrisera, Vännäs, SWEDEN). Cross-reacting proteins were revealed using peroxidase-conjugated secondary antibody (GE Healthcare Bio-Sciences Corp. Piscataway, NJ, USA) and the relative immunoresponse was estimated densitometrically as described by Retamal et al. [28].

3. Results

3.1. Changes in ATP and PPi hydrolysis

C. rotundus and *Z. mays* plants were submitted to drought stress and tonoplast vesicles were isolated by cell fractionation when the soil water reached 30 and 70% of deficit, and also after 24 h when the plants had been re-hydrated. Analysis of *C. rotundus* tonoplasts revealed that the ATP hydrolysis decreased progressively reaching 70% of inhibition upon the most severe stress (70% wd, Fig. 1A), while the PP_iase sustained high levels of PPi hydrolysis being inhibited by only 30%

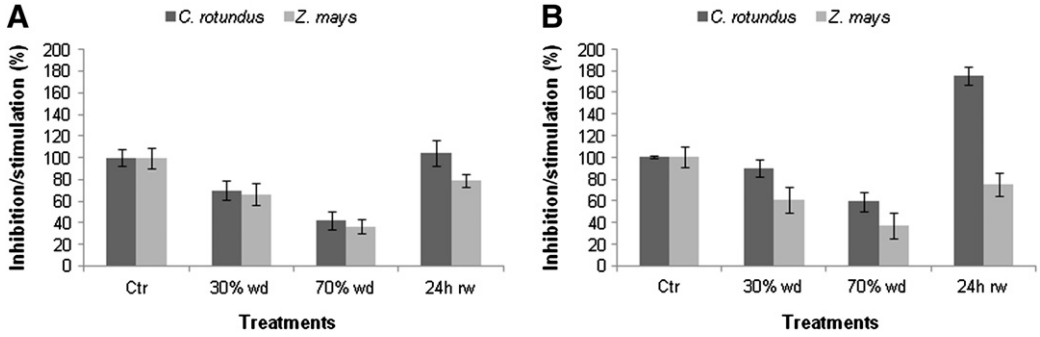

Fig. 1. V-ATPase (A) and V-PP_iase (B) hydrolytic activity in tonoplasts isolated from *C. rotundus* under increasing water deficit (wd) and 24 h after re-watering (rw). The reaction was started by addition of 1 mM ATP or 0.1 mM PP_i, at pH 7.0, and stopped after 30 min at 25 °C by addition of 5% TCA. Percentages were calculated from the specific enzymatic activities showed in Table 1. Values are means ± SE of eight independent experiments.

under high dehydration (70% wd, Fig. 1B). Moreover, the PP_i hydrolysis enhanced more than 2-fold in response to the relief of stress (24 h rw, Fig. 1B). Maize seedlings submitted to similar treatments were more sensitive to drought, with both ATP and PP_i hydrolysis decreasing progressively during the stress, and just recovering the control levels 24 h after rewatering (Fig. 1). Furthermore, the rates of ATP and PP_i hydrolysis were about 3-fold higher in tonoplast vesicles from *C. rotundus* plants (Table 1).

3.2. Proton pumping activities

Proton transport mediated by vacuolar H^+ pumps was analyzed using tonoplast vesicles isolated when the soil water reached 30 and 70% of deficit and 24 h after rewatering (Fig. 2). The H^+ pumping activities decreased along the stress, but this effect was more pronounced for the H^+-PP_iase. The tonoplast H^+ gradient generated by *C. rotundus* V-ATPase was inhibited by ~60% upon the most severe stress (wd 70%, Fig. 2A), while no PP_i-dependent H^+ pumping could be detected under this condition (Fig. 2B). Conversely, after re-watering, the initial rate of the PP_i-dependent H^+ transport activity was stimulated by ~50% when compared to the controls (Fig. 2B), while the V-ATPase activity was only barely affected (Fig. 2A). The H^+ gradient generated by the maize V-ATPase was also only slightly modified by drought stress (Fig. 2C), while the maize V-PP_iase showed a comparable profile to that of *C. rotundus* with a complete inhibition of H^+ pumping during severe stress (wd 70%) and an overactivation of PP_iase after re-watering (Fig. 2D). However, for the maize V-PP_iase the activation occurred only in the H^+ gradient formation, resulting in more than 6-fold increase in the pump coupling efficiency (Table 2). On the other hand, for the *C. rotundus* pump it was observed in a higher stimulation of the PPi hydrolysis over that found in its related H^+ pumping capacity (Figs. 1B and 2D).

3.3. Western blot analysis of the tonoplast proton pumps

The expression of the vacuolar H^+ pumps in the tonoplast under drought stress and after re-watering was evaluated using western blot analysis. As shown in Fig. 3A and C, V-ATPase immunoreactivity in *C. rotundus* vacuolar membrane increased by ~20% at the initial stage of the stress (30% wd), and then, the enzyme level reduced as the soil water deficit reached 70%. The protein content was recovered to the control levels after rehydrating (Fig. 3A and C). Interestingly, the level of H^+-PP_iase in *C. rotundus* increased ~80% when submitted to initial stress (Fig. 3B and D), but the immunoreactivity was restored to the control level during severe stress (70% wd), and remained unaltered even after 24 h rehydration (Fig. 3B and D). In *Z. mays*, a lower sensitivity was observed at the V-ATPase level in response to stress and rehydration (Fig. 3A). Contrasting with the stimulatory effect found in *C. rotundus*, *Z. mays* H^+-PP_iase immunoreactivity was reduced not only by 60% in the initial stress, but was also restored to the control level in the subsequent treatments (Fig. 3B).

Table 1
Comparison between the vacuolar proton pumps hydrolytic activities ($\mu mol \cdot mg^{-1} \cdot min^{-1}$) of *C. rotundus* and *Z. mays* under water deficit (wd) and 24 h after rewatering (rw). Values are means ± SE of eight independent experiments.

	ATP hydrolysis		PP_i hydrolysis	
Treatments	*C. rotundus*	*Z. mays*	*C. rotundus*	*Z. mays*
Control	1.73 ± 0.03	0.64 ± 0.014	1.31 ± 0.23	0.56 ± 0.009
30% wd	1.10 ± 0.03	0.41 ± 0.009	1.04 ± 0.06	0.31 ± 0.007
70% wd	0.70 ± 0.09	0.26 ± 0.008	0.74 ± 0.06	0.17 ± 0.004
24 h rw	1.74 ± 0.11	0.50 ± 0.008	1.75 ± 0.12	0.45 ± 0.015

4. Discussion

Plant distribution and abundance in natural environments and crop yield in agricultural systems are largely determined by water availability. Plants respond and adapt to water deficit at both the cellular and molecular levels, for instance by the accumulation of osmolytes and proteins specifically involved in stress tolerance. The plant vacuole is directly involved in this process as well as in ion signaling for drought responses [29]. In order to gauge whether the vacuolar H^+ pumps play a coordinated and active role on different mechanisms of plant drought adaptation, we compared the changes in the enzyme activities and expression occurring in *C. rotundus* L., a rustic stress-tolerant weed species, and in an agriculturally adapted crop variety (*Z. mays* L.).

The ATP hydrolysis (Fig. 1A) as well as the ATP-dependent H^+ pumping activity (Fig. 2A) of *C. rotundus* V-ATPase were inhibited in the same range during the stress, and after rewatering both activities also recovered their control levels. A close profile was observed in immunodetection assay (Fig. 3A) suggesting that such behavior reflects a modulation of the pump expression in the vacuolar membrane. This hypothesis is in line with previous findings indicating that whereas salinity had a minor effect on the expression of V-ATPase genes in *Arabidopsis thaliana*, heat and drought stress led to alterations in transcript amount and isoforms of its subunits [30]. However, such regulation seems to be characteristic of species adapted to stress like *C. rotundus* and *A. thaliana* [31], rather than of more sensitive cultivated species, like maize and tomatoes. Actually, it was shown that the message level for the catalytic (70-kDa) subunit A of the V-ATPase did not vary in tomato under drought stress [32]. Here, also no significant change was found in immunodetection of the subunit B in maize tonoplasts, whereas for *C. rotundus*, a clear decrease occurred under severe stress and a rapid recovery upon rehydration was observed (Fig. 3A).

On the other hand, the H^+-PP_iase hydrolytic activity of *C. rotundus* was barely affected by drought treatment (Fig. 1B), while its H^+ pumping was severely inhibited at this stress level (Fig. 2B). Upon 70% of water deficit no PP_i-dependent H^+ pumping was detected, but at the same condition (Fig. 2B) the PP_i hydrolysis was inhibited only by ~30% (Fig. 1B). This may indicate that the H^+-PP_iase is assuming an uncoupled functional conformation, since a possible membrane leakage

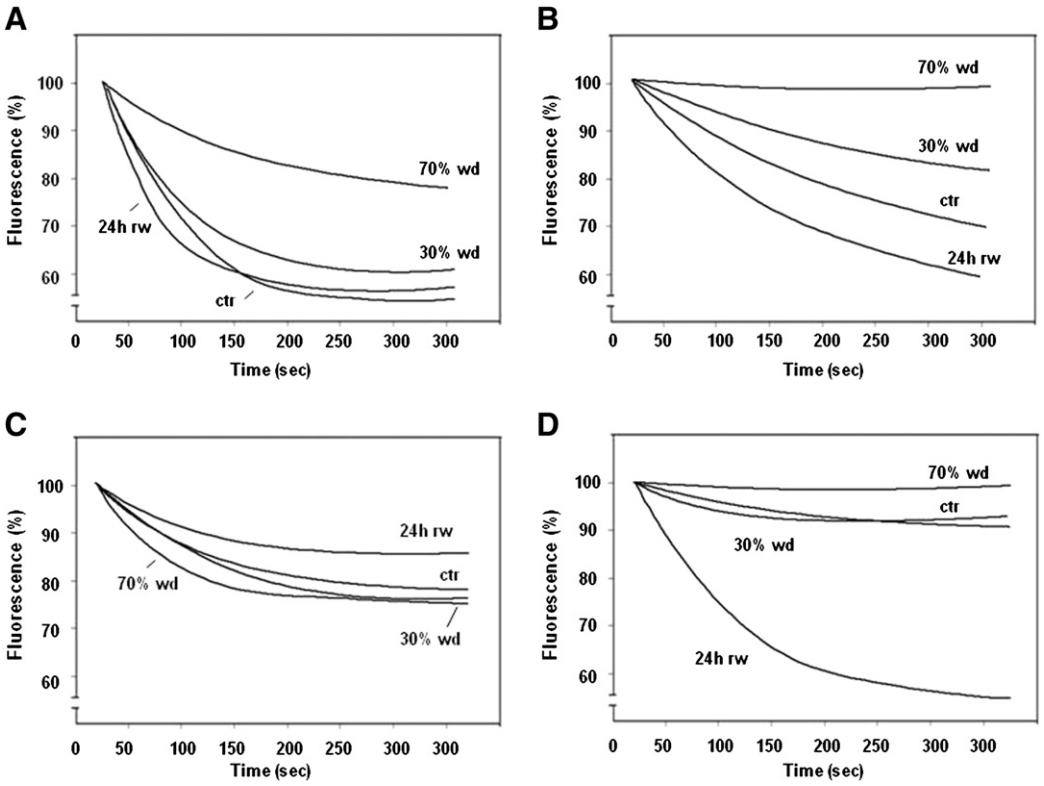

Fig. 2. Proton pumping activities of V-ATPase (A) and V-PP_iase (B) of tonoplast vesicles isolated from *C. rotundus* and V-ATPase (C) and V-PP_iase (D) from *Z. mays* seedlings irrigated daily (control, ctr), upon 30% or 70% of soil water deficit (30wd, 70wd), and 24 h after re-watering (rw). Proton translocation across membrane vesicles was monitored by the fluorescence quenching of ACMA in the presence of 50 μg protein of membrane vesicles; the reaction was started by addition of 1 mM ATP or 0.1 mM PP_i. The data are representative of three independent experiments.

could be ruled out taking into account that a significant ATP-dependent H^+ gradient was formed in the same tonoplast preparation (Fig. 2A). This possibility is reinforced by the data obtained 24 h after rewatering, where the PP_i hydrolysis enhanced ~110%, while the initial velocity of PP_i-dependent H^+ pumping increased only by ~60%.

It seems likely that the H^+-PP_iases could operate mainly as phosphate-recycling enzymes during severe drought stress, by undergoing an unusual and still unexplored uncoupling regulation. This possibility is especially interesting from the energetic viewpoint, because H^+-PP_iases use a simple, low-cost substrate pyrophosphate that is generated as a byproduct of several biosynthetic processes for macromolecules, such as protein, RNA and polysaccharides. Moreover, a single gene encoding a protein of ~80 kDa encodes the V-PP_iase and, theoretically, under metabolic stress, it should be easier and energetically safer to trigger its up-regulation than for the multimeric V-ATPase (~700 kDa). This is in line with the notion that for plants to react to their constantly changing environments and at the same time maintain optimal metabolic conditions, the expression, activity and interplay of the H^+ pumps have to be tightly regulated [33,34]. In this context, the present study also provides new insights on how transgenic plants that overexpress the H^+-PP_iases acquire a higher tolerance to P_i starvation [35], drought and salt stress [15,36–38] and capacity to nitrogen use efficiency [39]. Further studies are clearly required to better investigate the role of H^+-PP_iases as P_i-recycling enzyme active in Pi signaling, providing a complementary view on recent findings on phosphorus deficiency resulting in increased expression of the *AVP1* H^+-PP_iases and subsequent enhancement of the P-type plasma membrane H^+-ATPases [35].

Table 2
Comparison between the vacuolar proton pumps coupling ratio of *C. rotundus* and *Z. mays* under water deficit (wd) and 24 h after rewatering (rw). The H^+ pumps' coupling was calculated by the difference between the initial rates of H^+ pumping activity and the respective substrate hydrolysis. Values are means ± SE of three independent experiments.

	Coupling ratio			
	(V_0 H^+pumping/ATP hydrolysis)		(V_0 H^+pumping/PP_i hydrolysis)	
Treatments	*C. rotundus*	*Z. mays*	*C. rotundus*	*Z. mays*
Control	21.6 ± 2.2	29.1 ± 1.7	11.6 ± 1.2	10.6 ± 1.4
30% wd	28.9 ± 3.7	43.4 ± 7.8	7.54 ± 1.8	34.8 ± 2.1
70% wd	19.4 ± 1.8	104.0 ± 15.9	–	–
24 h rw	22.2 ± 2.9	23.8 ± 1.4	13.3 ± 1.2	70.4 ± 6.2

By overactivating their H^+-PP_iases, in the first drought stages and/or after the relief of stress, tolerant plant cells will decrease the ATP demand for maintenance of vacuolar electrochemical gradient, at the same time that the expression/activity of some uncoupled pumps will enhance P_i availability for the higher ATP synthesis and consume cytosolic PP_i required to overcome the constraints of transient stress exposures. Pyrophosphate is formed in a variety of biosynthetic reactions and needs to be promptly hydrolyzed in order to keep the overexpression of a myriad of stress responsive genes. This is also in line with a new emerging concept that in specific physiological conditions the role of H^+-PP_iase as a proton pump seems to be negligible [20,21]. These authors have provided very compelling evidences that during early seedling development, the major role of *Arabidopsis* H^+-PP_iase is the removal of the inhibitory metabolite PP_i rather than vacuole acidification. Previously, Hirono et al. [40] have also elegantly demonstrated, by random and site-directed mutagenesis, that discrete changes in specific amino acid residues dramatically affect the efficiency of H^+ translocation and energy coupling of the H^+-PP_iase of *Streptomyces coelicolor*. Thus, it is tempting to speculate that there could exist physiological ways to modulate the functionality of related residues in the plant enzyme, in response to several environmental stresses.

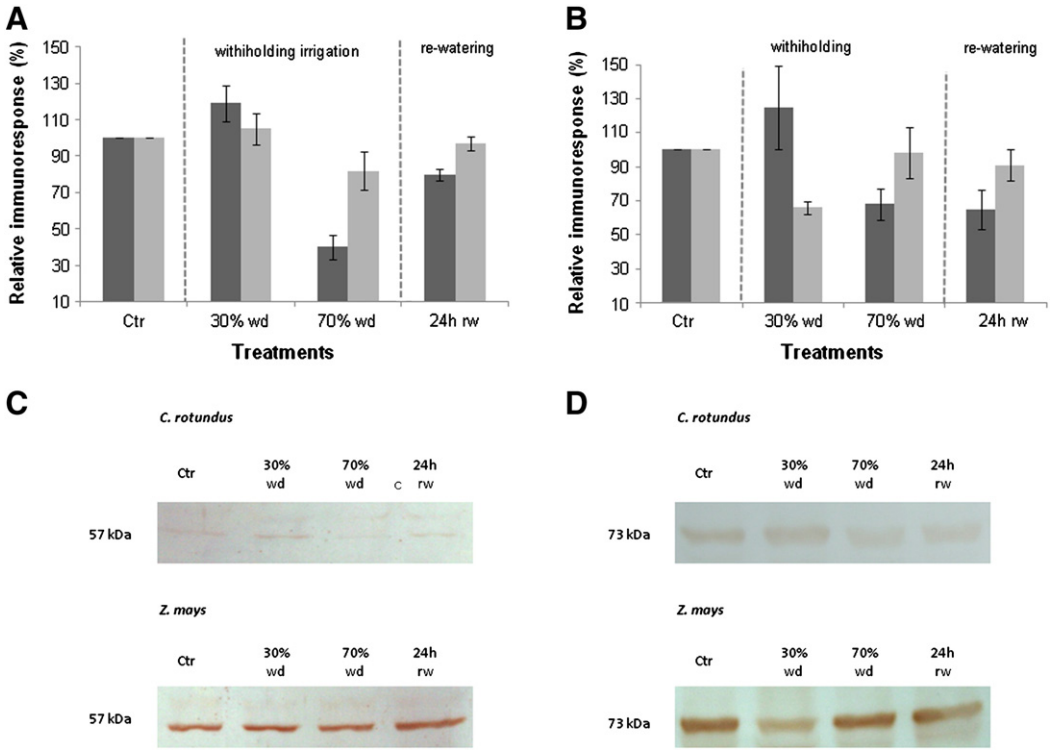

Fig. 3. Immunoblot analysis of V-ATPase subunit B and H^+-PP_iase under increasing water deficit (wd) and after re-watering (rw). Membranes were isolated from *C. rotundus* (dark bars) and *Z. mays* (light bars) plants as described in the Material and methods section, and submitted to immunoblot analysis. Western blots were used for quantification of the relative levels of the subunit B of V-ATPase (A and C) and H^+-PPase (B and D). The immunoresponse of control plants was considered as 100% in each case. The data are representative of three independent experiments.

In regard to the issue of biochemical adaptive differences developed between weeds and crops, it is worth noting the much lower hydrolytic activities of maize proton pumps in comparison to that of the weed. It seems likely that a high performance of the proton pumps can be one of the features developed by plants evolving natural environments, with the tonoplast electrochemical gradient being mainly sustained by a resistant V-ATPase and the H^+-PPiase assuming an uncoupled conformation towards warrant of the Pi supply and the PPi cytoplasmic control. On the other hand, cultivated crops were selected presenting high performance in agricultural fields based at least in part on the stability of their V-ATPase and on the capacity to strongly enhance the H^+-PPiase coupling, which assumes the main role in the vacuolar energization as soon as a stress condition is overcome. Taken together these results argue in favor of a reconsideration of the biochemical and physiological roles of the vacuolar proton pumps, with emphasis on the impressive H^+-PP_iase coupling plasticity and the respective relevance to the energy balance upon stress, not only for the vacuolar system but also for the cellular biosynthetic metabolism as a whole.

In conclusion, the lack of correlation between the PP_i hydrolysis, H^+ pumping activity data and protein expression in the tonoplast suggests a complex regulation of the H^+-PP_iases, which argues against a unique role as backup system to V-ATPases in maintaining the vacuolar H^+ gradient. From the physiological aspect, this study provides new perspectives to address the essential question of why H^+-PP_iase coexists with H^+-ATPase in the same vacuolar membrane. This work also proposes a new energetic adaptation characteristic of invasive weeds that enable them to disperse rapidly even into dry areas and outcompete crops and native vegetation.

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