Assessment of the requirement for aquaporins in the thylakoid membrane of plant chloroplasts to sustain photosynthetic water oxidation

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ABSTRACT

Oxygenic photosynthetic organisms use sunlight energy to oxidize water to molecular oxygen. This process is mediated by photosystem II complex at the lumenal side of the thylakoid membrane. Most research efforts have been dedicated to understand the mechanism behind the unique water oxidation reactions, whereas the delivery pathways for water molecules into the thylakoid lumen have not yet been studied. The most common mechanisms for water transport are simple diffusion and diffusion facilitated by specialized channel proteins named aquaporins. Calculations using published data for plant chloroplasts indicate that aquaporins are not necessary to sustain water supply into the thylakoid lumen at steady state photosynthetic rates. Yet, arguments for their presence in the plant thylakoid membrane and beneficial action are presented.

Keywords:
Aquaporin
Thylakoid membrane
Photosynthesis
Water oxidation
Water permeability

Highlights

1. Significant water amounts are consumed daily for photosynthesis in one chloroplast.
2. Simple diffusion may sustain water supply at steady state photosynthetic rates.
3. Low E_a of water transport indicate the presence of aquaporins in thylakoids.
4. They could contribute to fast regulatory volume changes of the thylakoid lumen.
5. Proteomic evidence of PIPs and TIPs in chloroplasts is worth further investigation.

Abbreviations: CHIP28, Channel-forming integral protein of 28 kD; Chl, Chlorophyll; E_a, Activation energy; MIP, Major intrinsic protein; P_d, Diffusional (basal) permeability in the absence of an osmotic gradient; P_f, Osmotic permeability; τ_a, Mean pre-exchange lifetime; PIP, Plasma membrane intrinsic protein; PS, Photosystem; SWC, Steady-state water consumption; TIP, Tonoplast intrinsic protein; WOC, water-oxidizing complex.

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1. Introduction

Molecular oxygen (O_2) has accumulated in Earth's atmosphere since Precambian, *i.e.*, some 2.4 billions years ago. This event, known as the Great Oxidation Event (GOE) [1] has forever completely modified life organization and development. Molecular oxygen is produced by oxygenic photosynthetic organisms (cyanobacteria, algae and land plants) that use sunlight energy to extract electrons from water and transfer them through the electron transfer chain to $NADP^+$, producing O_2 and NADPH (Fig. 1). NADPH together with ATP produced as a result of electron-coupled H^+ transport drive the fixation of CO_2 into carbohydrates. The electron and H^+ transfer reactions and ATP synthesis are mediated by four multiprotein complexes located in the thylakoid membrane, namely photosystem I and II (PSI and PSII), cytochrome b_6/f and the H^+-translocating ATP-synthase [2, 3]. These complexes are uniformly distributed in the thylakoid membrane of cyanobacteria, red algae, brown algae and diatoms. In land plants and green algae, PSII is restricted to cylindrical stacks of grana thylakoids, PSI and the ATP-synthase are only found in stroma-exposed regions of the thylakoid membrane, and cytochrome b_6/f is found in both types of membranes. Despite heterogeneity in organization and composition, the thylakoid network encloses a tiny but continuous aqueous space, named thylakoid lumen [4].

PSII is the first complex in the electron transfer chain since it performs the water oxidation reaction at the lumenal side of the thylakoid membrane (Fig. 1). The crystal structure of PSII from a cyanobacterium (*Thermosynechococcus vulcanus*) has been reported at a resolution of 1.9 Å [5]. At this resolution, information on the arrangement of protein subunits, cofactors and detailed structure of the water-oxidizing complex (WOC) was provided. This resolution also allowed the identification of more than 1,300 water molecules in each PSII monomer. Among these water molecules, four were found associated with WOC

and most likely serve as substrate for the oxidation reactions. A few water molecules were found within the membrane region, serving as ligands for chlorophyll (Chl) molecules. The remaining water molecules were distributed in two layers located at the stromal and lumenal surface of the PSII complex, with a larger proportion found at the lumenal side, where WOC is located. The binding and role of the bulk of water molecules is at present unknown. Due to the unique character and tremendous importance, most research efforts have been dedicated to understand the mechanism behind the unique water oxidation reactions, whereas the delivery pathways for water molecules into the thylakoid lumen have not yet been studied.

Water and solute permeability across membranes varies greatly among cell types and even within the same cell type [6]. The microinjection into *Xenopus laevis* oocytes of *in vitro*-transcribed CHIP28 RNA from human erythrocytes was the first demonstration of a facilitated diffusion of water since the expression of CHIP28 protein increased considerably the permeability of oocytes to water [7]. This discovery was awarded with Nobel Prize in Chemistry (2003) and was the beginning of the identification of membrane water channels (aquaporins) in all types of living organisms, ranging from bacteria to plants and animals [8]. These channel proteins belong to the major intrinsic protein (MIP) superfamily.

The presence of MIPs in the membrane can account for as much as 90 % of its water permeability [9]. The remaining permeability could be attributed to simple diffusion across cell membrane or passive transport through regions of membrane proteins possessing structural domains that could be permeated by water [10]. Using heterologous expression and transport assays, it has been shown that MIPs not only mediate water transport, but can also transport CO_2, H_2O_2, glycerol, urea, ammonia, boron, lactic acid, silicon, and arsenic (reviewed in [11]). The pore for transport of water and other small solutes is created between the six transmembrane helices. MIPs exist in the membrane as homotetramers, but the monomer is the functional unit. When it comes to the regulation of MIPs activity, a number of factors have been proposed, including cGMP, divalent cations (*eg*. Ca^{2+}, Mn^{2+}), pH, phosphorylation, and voltage dependence [12]. Recent studies point to new modes of regulation for PIPs, namely by auxin-mediated trafficking between plasma membrane and cytoplasm, indicating a dynamic control of their density in membranes [13, 14]. Most recently, interaction of Arabidopsis PIPs with TIPs has been demonstrated when expressed in yeast and proposed to play a role in their targeting and activity regulation in plants [15].

MIPs appear to be involved in numerous physiological processes in living organisms. They have also been characterized in plants, and shown to play multiple roles in cellular and water plant balance, nutrient acquisition, membrane biogenesis, and adaptation to stress [16-

18]. New roles for aquaporins are also emerging such as dynamic equilibration and subcellular partitioning of their various substrates and a contribution to cell expansion and possibly cell division. Below, the recent experimental evidence for the presence of MIPs in cyanobacterial and plant chloroplast membranes is reviewed.

Cyanobacterial cells resemble chloroplasts of higher plants in terms of organization [19], membrane lipid composition [20, 21], and also structure of the photosynthetic machinery [22]. According to the endosymbiotic theory, the cyanobacterial plasma and thylakoid membrane correspond to the chloroplast inner envelope and the thylakoid membrane, respectively [23]. Based on genomic data available in Cyanobase (http://genome.kazusa.or.jp/cyanobase) aquaporins are present in cyanobacteria. The *aqpZ* gene in *Synechocystis* sp. PCC 6803 encodes a water channel protein, which is homologous to the *aqpZ* gene product from *Escherichia coli.* The Synechocystis protein has been shown to facilitate water transport across plasma membrane and to play a role in protection against hyperosmotic stress [24, 25]. Thus far AqpZ has been found using proteomics in Synechocystis plasma membrane [26]. In the same report, proteomics of Synechocystis thylakoid preparations have also been presented, but no aquaporins have been identified.

The MIP superfamily in the model plant *Arabidopsis thaliana* has 35 members [27]. Among Arabidopsis MIPs, there are 13 plasma membrane intrinsic proteins (PIPs) and 10 tonoplast intrinsic proteins (TIPs). Three Arabidopsis TIPs, namely TIP1;1, TIP1;2 and TIP2;1 have been detected by Ferro et al. [28] using the proteomic approach in the chloroplast envelope of Arabidopsis, but their presence has been regarded as a possible vacuolar contamination. In another proteomic study of the Arabidopsis chloroplast [29], several PIPs (PIP1;2, PIP1;3, PIP2;1 and PIP2;2) and TIPs (TIP2;1 and TIP2;2) were identified in the envelope fraction, but their presence was considered insecure since possible contamination with plasma and vacuolar membranes could not been excluded. Zybailov et al. [30] have detected TIP2;1 in the thylakoid fraction, but no other isoforms. In a more recent study, Ferro et al. [31] identified again TIP1;1, TIP1;2 and TIP2;1 in chloroplast membranes from Arabidopsis. TIP1;1 was detected only in the chloroplast envelope, and has been considered as a contaminant because in the same fraction, the vacuolar marker V-ATPase has also been detected. TIP2;1 and TIP1;2 proteins were found in both thylakoid and envelope membranes although in different ratios. Because V-ATPase was not detected in the thylakoid fraction, at least these two TIPs were considered as thylakoid proteins. In the same report, peptides for some PIPs (PIP1;2, PIP1;3, PIP2;4 and PIP2;7), were also detected in envelope membranes preparations. Although the plasma membrane marker H^+-ATPase was also found in those

preparations, a genuine chloroplast location for one or several isoforms of the PIP family was proposed. Most recently, TIP1;1 and PIP2;1 (also named PIP2A) were identified by mass spectrometry in the Arabidopsis envelope fraction [32].

A dual location of some PIPs and TIPs would not be surprising taking into consideration the case of AQP1 from *Nicotiana tabacum,* which localized to the plasma membrane and also to the chloroplast inner envelope [33, 34]. Interestingly, the substrate specificity was found to be location-dependent since only the plasma membrane AQP1 transported water, whereas the envelope AQP1 facilitated diffusion of CO_2, although the requirement for CO_2 aquaporin in biological membranes is still controversial. Taken together, the reports reviewed above provide indications for the presence of TIPs and PIPs in chloroplast membranes (illustrated in Fig. 1), but their location within the chloroplast, substrate specificity and physiological role(s) need further investigations.

In this Hypothesis paper, we attempt to answer the question whether the diffusional (basal) water permeability of the thylakoid membrane provides sufficient rates of water transport to fulfill the requirements of WOC at the lumenal side of PSII complex, and to be used as a source of electrons in the photosynthetic electron transport chain. Alternatively, water molecules need to cross the membrane *via* aquaporins to fulfill the photosynthetic requirements. These hypotheses need to be experimentally verified in the near future.

2. Analysis of photosynthetic consumption of water and its availability in the thylakoid lumen

To estimate the amount of water consumed during photosynthetic reactions and the amount available inside the chloroplast stroma and the thylakoid lumen, we have used data extracted from the literature for dark-adapted leaf chloroplasts (Table 1) and performed the calculations described below.

2.1 The maximal amount of water consumed daily for photosynthesis within one chloroplast

The daily photosynthetic water consumption was estimated in two ways: a) based on the O_2-evolving activity in isolated thylakoid membranes, and b) based on the O_2-evolving activity in intact leaves.

a) The steady state rate of net O_2 evolution measured in spinach thylakoid membranes using Clark-type electrode and saturated white light was approx. 200 μmol (mg Chl)$^{-1}$ h^{-1} (Table 1).

Based on this and taking into account that two molecules of water are oxidized to release one molecule of O_2, a value of 400 μmol (mg Chl)$^{-1}$ h^{-1} was obtained for the rate of water consumption to sustain steady-state oxygen evolution (SWC). Assuming that a plant is exposed to sunlight during one day for up to 16 h, the estimated daily SWC is 6.4 mmol (mg Chl)$^{-1}$. Based on this activity and knowing the volume occupied by chloroplasts corresponding to 1 g Chl (Table 1), we determined that 6.4 mmol water are consumed daily from 36 x 10^{-9} m^3 of chloroplast. Based on the volume of one chloroplast (Table 1), a value of 5.9 pmol can be calculated for the daily SWC of one chloroplast. As described below, the same result was obtained when performing calculations using data for the thylakoid lumen, where the water oxidation takes place. Based on the volume of thylakoid lumen (g Chl)$^{-1}$ (Table 1) a value of 2.3 x 10^{-9} m^3 (mg Chl)$^{-1}$ was obtained. This means that a 6.4 mmol of water is consumed daily from 2.3 x 10^{-9} m^3 of the thylakoid lumen. Based on the volume of the thylakoid lumen of one chloroplast (Table 1), it was calculated that 5.9 pmol water are consumed daily within the thylakoid lumen of one chloroplast.

b) The steady state rate of net O_2 evolution measured in intact leaves using saturated white light per m^2 of leaf was approx. 50 μmol m^{-2} s^{-1} (Table 1). If considering that in addition to photosynthetic O_2 evolution, various O_2-consuming processes take place in illuminated leaves (oxygenation reaction by Rubisco, Mehler reaction and mitochondrial respiration), a value of approx. 80 μmol m^{-2} s^{-1} was estimated using isotope techniques for the steady state rate of gross O_2 evolution (Table 1). Based on this gross rate, a daily SWC of 9.2 mmol m^{-2} was obtained. Knowing the Chl content of a leaf (Table 1), a daily SWC of 18.4 mmol (mg Chl)$^{-1}$ was determined. Using data for Chl content from Table 1, the daily SWC of one chloroplast was 16.5 pmol. This last value, although of the same magnitude order, is approx. three fold higher than the one obtained based on net O_2-evolving activity of isolated thylakoid membranes (see above). There could be multiple reasons for variation and lower activity of thylakoids including the isolation procedure, assay method, and that chloroplast stroma components are required for full activity. In section 4, we have taken into consideration the results of gross O_2-evolving activity in leaves.

2.2 The maximal amount of water inside one chloroplast

Based on the volume of one chloroplast and the volumes occupied by the thylakoid membrane and its lumen (Table 1), the volume of chloroplast stroma is calculated as [33-(4.1+2.1)] x 10^{-18}= 26.8 x 10^{-18} m^3. Knowing the molar volume of water (18 x 10^{-6} m^3 mol^{-1}) and assuming that the entire volume of stroma is occupied by water, the maximal amount of water present in

the stroma of one chloroplast is 1.5 pmol. Based on the volume of the thylakoid lumen in one chloroplast (Table 1), and assuming that the entire volume of lumen is occupied by water, the maximal amount of water inside the thylakoid lumen of one chloroplast is 0.1 pmol.

The results of our calculations indicate that the amounts of water present within the stroma and the lumen are about 4- and 60-fold, respectively, lower than SWC when calculated based on data from thylakoids, and about 10- and 170-fold, respectively, lower, if taking into account data from leaves (section 2.1). In other words, it is estimated that using SWC of leaves the thylakoid lumen could be emptied in approx. 4 min. Consequently, there is great demand for water resupply from the chloroplast to sustain SWC. In addition, it is also of great importance to rapidly resupply water to the chloroplast from the cytosol since it has been shown that photosynthetic water oxidation is influenced by changes in the chloroplast volume [35]. To assess if water cross the thylakoid membrane by simple diffusion and/or diffusion facilitated by aquaporins, below we analyze the water permeability of the thylakoid membrane and compare it with that of membranes known to harbor MIPs and that of aquaporin-free lipid bilayers.

3. Analysis of water permeability of the thylakoid membrane

Water permeability of any membrane or cell can be described using two parameters, *i.e.* the diffusional (basal) permeability (P_d) and the osmotic permeability (P_f). P_d is determined by monitoring the exchange of labeled water molecules between the two sides of the membrane in the absence of any driving force (osmotic gradient), whereas P_f relies on the measurement of volume changes due to water exit or entry, depending on the direction of the osmotic gradient.

For a cell, $P_d = V/(A\ \tau_a)$ [36] (Eq. 1) ,

where A and V represent the surface area and internal volume of the cell, respectively, and τ_a is the mean pre-exchange lifetime of water inside the cell. If intracellular water diffusion is very slow, then the actually passage through the membrane may not be the limiting step in water transport, leading to underestimation of P_d. Thus, equation 1 actually estimates the apparent P_d' since the dependence of water permeability on the intracellular water motion is unknown.

To determine P_f, the rate of volume change must be measured and computed using the following equation:

$$dV_{(t)}/dt = (P_f)(SAV)(MVW)(C_{in}/V_{(t)} - C_{out})\ [37] \quad \text{(Eq. 2)},$$

where $V_{(t)}$ is the relative volume of the cell at time *t,* (*i.e*., volume at time *t,* divided by the initial volume), SAV is the surface area to volume ratio, MVW is the molar volume of water (18×10^{-6} m^3 mol^{-1}), C_{in} and C_{out} are initial solute concentrations inside and outside the cell.

When comparing the values for P_d and P_f, they are often not found equal. A P_f/P_d ratio >1 indicates facilitated diffusion involving the activity of water channels due to their high P_f [36, 38]. For example, the well-studied erythrocyte plasma membrane, having a high density of water channels, has a P_f/P_d ratio of about 7 [39]. Although water channels have been less studied in plants, a similar P_f/P_d ratio has been found for the root tonoplast and a lower one (about 3) for the root plasma membrane [40]. The high P_f/P_d ratio was attributed to a much higher P_f determined for the tonoplast than for the plasma membrane [41]. However, later studies indicated similar P_f values for the two types of membranes [42, 43], emphasizing the importance of the isolation procedure for plasma membrane vesicles. P_f/P_d ratio ~ 1 is usually found for artificial lipid bilayers devoid of water channels [44]. In addition, water diffuses through lipid bilayers with high activation energy (E_a > 10 kcal mol^{-1}, Table 2). For comparison, the erythrocyte and also the plant tonoplast and plasma membrane have E_a ~ 5 kcal mol^{-1} (Table 2 and refs. [39, 41]).

3.1 The diffusional water permeability of the thylakoid membrane

As compared to available experimental data on P_d and P_f for artificial lipid bilayers, erythrocyte and plant plasma membranes and tonoplast (Table 2 and ref. [40-43]), to our knowledge, there is no measure of these two water permeability parameters for thylakoids. Nevertheless, the τ_a parameter for mean lifetime of water inside the thylakoid lumen has been measured using NMR by two independent laboratories [45, 46]. Introducing the τ_a values (1-20 ms) into equation 1 together with the values for the volume of thylakoid lumen in dark-adapted chloroplasts and for the total thylakoid membrane area (grana+stroma, Table 1), an apparent P_d' ranging from 0.02×10^{-5} to 0.4×10^{-5} m s^{-1} was obtained. It is important to recall from Introduction that although the lumen is a continuous space, the thylakoid membrane is a network composed of cylindrical grana stacks interconnected by stroma-exposed regions. Thus, roughly half of the thylakoid surface is in direct contact with the stroma, *i.e.* (5.4-1.4) x 10^{-10}= 2.4×10^{-10} m^2, hence available for water transport. Based on this consideration, the recalculated P_d' values for thylakoids will range between 0.045×10^{-5} and 0.9×10^{-5} m s^{-1}.

Using τ_a values given in Table 2, volumes and areas from the literature [47, 48], the apparent P_d' for the chloroplast envelope and the erythrocyte plasma membrane were also calculated, and found to be only slightly lower than the experimentally determined P_d values for the same type of membranes (Table 2). This indicates that calculation of P_d' provides data that are reliable and useful for comparative studies in the absence of experimentally determined P_d.

The P_d' values obtained here for thylakoids are in the same range as the P_d values for artificial lipid bilayer or chloroplast envelope and much lower than the ones reported for the aquaporin-rich erythrocyte membrane (Table 2). Moreover, P_d' for thylakoids is similar to P_d for erythrocyte membranes totally deficient in aquaporins (0.8×10^{-5} m s^{-1} [39]), suggesting that thylakoid membranes form a significant barrier to water flow. Presence of aquaporins would be helpful here if bulk water flow is required during regulatory volume changes. However under steady state conditions diffusional water flux may be sufficient to sustain the metabolic activities of the thylakoid.

3.2 The osmotic water permeability of the thylakoid membrane

The P_f parameter has not yet been determined experimentally for either chloroplasts or thylakoids, and its estimation is not as straightforward as in the case of P_d. Data available in the literature (Table 1) usually describe dark-adapted chloroplasts and thylakoids. In other words, we do not have kinetic data for lumenal V_t, thylakoid SAV, C_{in} and C_{out} when an osmotic shock is applied, to estimate P_f using equation 2. If aquaporins are present in the thylakoid membrane, then P_f is expected to be several-fold higher than P_d, as in the case of other aquaporin-containing membranes [39, 40]. This possibility remains to be investigated.

4. Comparison of water permeability parameters of the thylakoid membrane with water oxidation requirements

4.1 Comparison of P_d' with SWC

Since P_f could not be estimated, the question we address below is whether P_d' could account for the water resupply to the thylakoid lumen to fulfill the photosynthetic requirements. The daily SWC in the thylakoid lumen of one chloroplast (based on the gross O_2-evolution of leaves) is approx. 16.5 pmol (section 2.1). Assuming that there are no water channels (P_f=P_d) the gradient required for daily water resupply and maintenance of a constant volume can be computed using the following simple equation:

$$\text{Flux} = P_f \text{ x surface area x osmotic gradient} \quad \text{(Eq. 3)}$$

Using the determined P_d' (Table 2) as P_f, and the surface area of thylakoids in one chloroplast exposed to stroma (section 3.1), we calculated that the required osmotic difference between the thylakoid lumen and the stroma would be ranging between 0.15 mM and 3 mM. These osmotic gradient values are very small to drive significant water flux across a membrane in the absence of very high density of aquaporins, as seen in kidney collecting duct with high water reabsorbtion [49]. Since steady state photosynthetic rates do not require large water influx, the presence of aquaporins to facilitate water diffusion may not be necessary.

4.2 Comparison of τ_a with SWC

Below we compare the number of water molecules consumed in SWC with the pre-exchange lifetime (τ_a) of water molecules inside the thylakoid lumen. The daily SWC of one chloroplast (16.5 pmol) was converted using Avogadro's number (6.022×10^{23}) to 1.8×10^8 water molecules consumed per second. This value should be now compared with the number of water molecules present in the thylakoid lumen, assuming that its entire volume is occupied by water (section 2.2, 0.1 pmol • $6.022 \times 10^{23} = 6 \times 10^{10}$). An average pre-exchange lifetime of water in the thylakoid lumen of 10 ms (based on τ_a values ranging between 1-20 ms, Table 2) means that its content is exchanged 100 times every second, and an activity of 6×10^{12} molecules exchanged per sec can be estimated. This is about 3×10^4 fold faster than SWC when expressed per second. This number could be largely overestimated due to the assumption of thylakoid lumen fully occupied by water. Nevertheless, it becomes obvious that water oxidation is not the primary reason for the high rates of water exchange across thylakoid membrane, which instead may have other roles for the optimal function of the thylakoid, as discussed below.

5. Could aquaporins be beneficial for thylakoid membranes?

The most convincing argument for the activity of aquaporins in the thylakoid membrane is the low E_a of ~5 kcal mol^{-1}. If aquaporins are not necessary to sustain SWC, then the question is what could be the benefit for the chloroplast thylakoids in harboring such proteins with high rates of water exchange?

To clarify the physiological relevance of aquaporins in well-studied biological membranes such as the plasma membrane of erythrocyte cells, two hypotheses have been

proposed by Hill and coworkers [9]. In the simple permeability hypothesis, the role of aquaporins is to increase the water flow rates by raising the osmotic permeability of the membrane above that of artificial lipid bilayers. The fact that aquaporins increase membrane permeability is widely proven, but does not explain many observations at the cellular, subcellular and tissue levels, for example, the lack or partial effect on water permeability in knockouts or knockdowns of aquaporins. The second proposed hypothesis by Hill and coworkers was that aquaporins are osmotic or turgor sensors, *i.e.* transmembrane sensors of difference in osmotic or turgor pressure through changes in the structure of the monomers within the tetramer configuration. Although there is no direct experimental evidence, this is an interesting possibility, which could explain many basic processes where aquaporins have been implicated. It has been recently shown that in case of flooding in plants, pH changes in the cytosol lead to simultaneous closure of plasma membrane aquaporins [50]. Other studies have shown that aquaporins are involved in cell volume regulation in response to osmotic stress in cyanobacteria [25] and yeast [51], and are also expected to play this role in plant cells [9].

Thylakoids have a low apparent P_d' so that having aquaporins could be a clear advantage in increasing water permeability even if not needed for water oxidation. The role of thylakoid aquaporins could be to sustain net flux of water movement at a rate suitable for fulfilling other functions. Recently, it has been shown that the thylakoid lumen of dark-adapted protoplasts can expand to a double volume following light exposure [52]. In the same report, several benefits have been proposed for the observed expansion of the lumen, such as facilitating diffusion of mobile electron carriers between cytochrome b_6/f and PSI, and repair of photoinactivated PSII complexes. The mechanism behind the observed regulatory changes in the volume of the lumen is largely unknown. Nevertheless, there is experimental evidence for extensive light-dependent and dark-reversible ion fluxes across the thylakoid membrane, specifically Cl^- uptake into the lumen, which was proposed to maintain electroneutrality during photosynthetic H^+ uptake [53]. Large influx or eflux of Cl^- ions and other solutes may happen in very short time scales (seconds to minutes). To explain the observed increase in volume of the thylakoid lumen [52], the Cl^- flux must be accompanied by movement of large amounts of water upon exposure to light or removal of light. The rate of volume changes may vary according to the light intensity, and therefore the water influx should be regulated. Simple diffusion is not regulated in a manner that will contribute to the maintenance of water homeostasis in the chloroplast. Furthermore, this mode of water transport cannot account for a rapid, regulated and selective water movement across thylakoids during exposure to light.

Aquaporins represent a special pathway with the above-listed characteristics. Taken together, the presence of aquaporins in the thylakoid membrane could indeed be beneficial for rapid volume changes of the lumen. Notably, volume changes have also been reported for chloroplasts. More specifically, they can expand in volume or shrink in response to various treatments, such as low temperature, light, salt, heat and osmotic stress [35, 54-57]. This is in support of the requirement of aquaporins in the envelope membranes of chloroplast. Thus, the proteomic evidence for existence of aquaporins in the chloroplast (reviewed in section 1) may not be a preparation artifact, but convincing results worth further experimentation.

Although not directly related to the beneficial contribution of aquaporins in the thylakoid membrane, an interesting question is why would the increase in volume of the thylakoid lumen [52] only happen in certain conditions such as light exposure. Some considerations are summarized below. First, measurement of the ion content for dark-adapted chloroplasts indicated K^+ being the major monovalent cation (160-200 mM), which could be replaced by Na^+ (40-70 mM), and Cl^- as the major anion (100 mM) [35]. In the same report, the major fraction of these ions was found in the stroma since only 10 %, 5-10 % and 10-20 % of the above concentrations of K^+, Na^+ and Cl^- were found inside the thylakoid lumen. Even though information about concentration on other osmolytes in the stroma (*e.g.* sugars) is not considered here, it is obvious that in dark-adapted chloroplasts the direction of the osmotic gradient would not favor net flux of water movement from the stroma into the thylakoid lumen. Since increase in thylakoid volume has been observed in the light, we suggest that this is due to massive water flow into its lumen following influx of Cl^- ions in response to lumen acidification [53], and possibly also influx of other yet unknown osmolytes. This would mean that the direction of the osmotic gradient in the light has been reversed as compared to the dark state. The molecular mechanism of water movement across the thylakoid membrane may be more complex than originally anticipated and requires detailed investigation.

6. Concluding remarks

Significant amounts of water are consumed daily in one chloroplast during photosynthetic reactions (60-170 fold of its thylakoid lumen volume), and water must be continuously supplied to maintain constant volume and sustain SWC. Although the calculated P_d' of the thylakoid membrane is similar to that of an erythrocyte plasma membrane deficient in aquaporins, it may provide sufficient rates of water flow to sustain SWC. Thus, the activity of

aquaporins may not be necessary for this process in thylakoids. Nevertheless, the fast water exchange indicated by τ_a and specially the E_a value reported in the literature for thylakoids, which is as low as that for the erythrocyte plasma membrane, argue for potential presence of aquaporins. Their beneficial contribution is postulated in relation to recently reported fast regulatory volume changes of the thylakoid lumen in the light. To date, no chloroplast-specific aquaporins have been identified. However, large-scale analysis of the chloroplast proteome indicated that several members of the PIP and TIP families are located in chloroplast membranes. Validation of their location and understanding their role in chloroplast membrane permeability must be the focus of future work.

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TABLES

Table 1

Parameters used to assess the amount of water available and consumed during photosynthetic reactions.

Parameter	Value	Reference
Steady state net O_2 evolving activity of thylakoids	125 μmol (mg Chl)$^{-1}$ h^{-1} 217 μmol (mg Chl)$^{-1}$ h^{-1}	[58] [59]
Steady state net/gross O_2 evolving activity of intact leaves	47-52 μmol m^{-2} s^{-1} /80 μmol m^{-2} s^{-1}	[60, 61]
Volume of chloroplasts corresponding to 1 g Chl	36 μm^3 g^{-1} Chl	[47]
Leaf Chl content	0.5 g m^{-2}	[47, 62, 63]
Volume of one chloroplast	3.3 x 10^{-17} m^3	[47]
Volume of the thylakoid lumen corresponding to 1 g Chl	2.3 x 10^{-6} m^3 g^{-1} Chl	[47]
Volume of thylakoid membranes (exclusive lumen) in one chloroplast	4.1 x 10^{-18} m^3	[47]
Volume of thylakoid lumen in one chloroplast	2.1 x 10^{-18} m^3	[47]
Chlorophyll content in one chloroplast	9 x 10^{-13} g	[47]
Total area of grana membranes per chloroplast	3.0 x 10^{-10} m^2	[47]
Total thylakoid area (grana+stroma) per chloroplast	5.4 x 10^{-10} m^2	[47]
Maximum amount of water consumed daily within the thylakoid lumen of one chloroplast (based on thylakoid/leaf O_2 evolving activity)	5.9 pmol/16.5 pmol	This work
Maximum amount of water present in the stroma of one chloroplast	1.5 pmol	This work
Maximum amount of water present inside the thylakoid lumen of one chloroplast	0.1 pmol	This work

Table 2.

Parameters of water permeability for various types of biological membranes.

Type of membrane	P_d **(m s^{-1})**	P_d **' (m s^{-1})[a]**	P_f **(m s^{-1})**	***Ea*** **(kcal mol^{-1})**	τ**a (ms)**	**References**
Artificial lipid bilayers	0.5 x 10^{-5} to 7.3 x 10^{-5}	n.d.	1.7 x 10^{-5} to 10^{-4}	10-17	0.015-0.05	[64-66]
Erythrocyte plasma membrane	1.8 x 10^{-5} to 8.4 x 10^{-5}	2.3 x 10^{-5} to 6.1 x 10^{-5}	2.3 x 10^{-4} to 10^{-3}	~ 5	10-17	[39, 48, 67-69]
Chloroplast envelope	0.9 x 10^{-5}	0.1 x 10^{-5} to 0.4 x 10^{-5}	n.d.	n.d.	88-320	[70, 71]
Thylakoid membrane	n.d.	0.045 x 10^{-5} to 0.9 x 10^{-5} m s^{-1}	n.d.	~ 5	1-20	[45, 46]

[a]Estimated in this study.

Figure legends

Fig. 1. Water transport across chloroplast membranes and its use during photosynthetic reactions. Water is transported into the chloroplast stroma across the envelope membrane and further on across the thylakoid membrane into the lumen. Whether this process takes place by simple diffusion and/or diffusion facilitated by aquaporins is currently unknown. Several plasma membrane- and tonoplast-intrinsic proteins (PIPs, TIPs) have been identified by proteomics in the chloroplast inner envelope and the thylakoid membrane, but their chloroplast location and role in the chloroplast require further investigation. Water is used by photosystem II (PSII) complex harboring the water-oxidizing complex (WOC) at the lumenal side of the thylakoid membrane. With the help of WOC, PSII extracts electrons from water and donates them to electron carriers bound to the cytochrome b_6f (cyt b_6f) and then to PSI with $NADP^+$ as the final acceptor. H^+-coupled transport across the thylakoid membrane drives ATP synthesis.