A NEW FLUORESCENCE-BASED METHOD TO MONITOR THE pH IN THE THYLAKOID LUMEN USING GFP VARIANTS

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Summary

The Δ pH-dependent/Tat pathway is unique for using only proton motive force for driving proteins transport across the thylakoid membrane in chloroplasts among five different pathways. 9-aminoacridine fluorescence quenching is widely used to monitor the Δ pH developed across the thylakoid membrane in the light. However, this method suffers from limited sensitivity to low Δ pH values and to spurious fluorescence signals due to membrane binding. In order to develop a more sensitive method for monitoring the real pH of the thylakoid lumen without these problems we transformed *Arabidopsis thaliana* with a ratiometric pH sensitive GFP variant (termed pHluorin) targeted to the lumen by the prOE17 transit peptide. Positive transgenic plants displayed localization of pHluorin in the chloroplast by confocal microscopy, and fractionation experiments revealed that it is in the lumen. The pHluorin signal was the strongest in very young plants and diminished as the plants matured. The pHluorin released from the lumen displayed the expected fluorescence intensity changes in response to pH titration. The fluorescence signal in isolated chloroplasts responded to illumination in a manner consistent with light-dependent lumen acidification. Future experiments will exploit the use of this new pH-indicating probe of the thylakoid lumen to examine the influence of the thylakoid Δ pH on ATP synthesis and protein transport.

Key words: Chloroplast, GFP, Genetically encoded biosensor, thylakoid lumen pH, proton dynamics

1. Introduction

Chloroplasts are most prominent organelles in green plants that are responsible for the reactions of photosynthesis through which most of the planet's reduced carbon and atmospheric O₂ are produced. In the light-dependent reactions, chloroplasts convert sunlight into chemical energy through the processes of electron transport and photophosphorylation. By the mid-1970s most researchers in bioenergetics accepted the basic tenets of the chemiosmotic coupling hypothesis, which states that the energy required for ATP synthesis was transiently stored in an electrochemical proton gradient across the energy-transducing membrane [1, 2]. Despite its stunning success, a number of labs question the classic chemiosmotic theory on whether the protonmotive force (pmf) arose from protons delocalized throughout the bulk aqueous volume or from protons located at the membrane surface [3].

As well studied, nucleus-encoded chloroplast proteins are synthesized in precursor form and import into the chloroplast via Toc and Tic complex [4]. Stromal chaperones and the stromal processing peptidase cleave the transit peptide from the incoming precursor protein driven by nucleotide triphosphate (NTP) hydrolysis. Then lumen located protein continues their step across the thylakoid membrane process via four different pathways: SRP (signal recognition particle) machinery, spontaneous, Sec and Tat pathways [4-6]. Precursors are recognized by the specific transport machinery through typical transit peptides. Not only native proteins but also foreign proteins fused by the transit peptide can be located to organelle on purpose. It was reported that targeting of EGFP within chloroplasts by cpTat pathway was successful [7].

Since 1966 originated from *Aequorea Victoria*, the wild type GFP cDNA has been modified by codon usage and removal of cryptic intron, to get higher intensity, improved solubility, less temperature sensitivity and different spectral characteristics for plants [8]. Engineered GFP protein is now widespread for plants (Av-GFP) for cellular localization, hormone movement, protein interaction, and so on [9-11]. Fluorescence intensity is also a useful indicator for comparing gene

expression and activity of promoters or enhancers, cellular or extracellular ion dynamics [12]. Especially after pHluorins were engineered [13], it becomes more popular to measure proton activity and cytoplasmic free Ca concentration in root, pollen or cytoplasm under stress [8, 14, 15]. Ratiometric pHluorin is a pH-sensitive variants of Av-GFP, containing eight amino acid mutations (E132D, S147E, N149L, N164I, K166Q, I167V, R168H and L220F). The protein has double excitation wavelength at 395nm and 475nm, displaying a reversible excitation ratio shift between pH 7.5 and 5.5 [13], So we think about locate this ratiometric pHluorin to the thylakoid lumen.

Previous measurements relating the Δ pH component of the pmf to protein transport on the cpTat pathway were made using 9-aminoacridine (9-AA) fluorescence as readout of the Δ pH . However this assay is insensitive to values of the pH gradient lower than approximately 1.8 pH units and to spurious fluorescence signals due to membrane binding [16]. In an effort to develop a more sensitive assay for the thylakoid transmembrane Δ pH, we constructed a novel pH-sensitive GFP variant by overlapping the first 1-395 bp of sGFP (containing increasing solubility) with the last 324 bp of ratiometric pHluorin containing all of the pH sensitive mutated amino acids together to get a new soluble and high quantum efficiency fluorescent protein. This modified ratiometric pHluorin was then driven by 35S promoter and targeted to chloroplast lumen by fusing proe17 transit peptide before its cDNA. A series of experiments were performed to characterize the phenotype of the transformed *A. thaliana* and to demonstrate the proton gradient by lumen localized pHluorin. Fluorescence images showed that pHluorin indeed locate at the thylakoid lumen. Luminal protein was released to prove that pHluorin was soluble in the lumen and responded to different pH. Confocal image was applied on mesophyll cells and guard cells of transformats during photosynthesis, to show the changes of fluorescence signal responding to photosynthetic acidity. Consequently, it provides the possibilities to apply this novel approach in the aspects of pmf in the chloroplast lumen. We also discussed the potential problems that may encounter when using this probe to do measurement.

2. Materials and Methods

2.1 Plasmid Constructs and Transformation

Ratiometric pHluorin gene was provided by the Memorial-Sloan Kettering Cancer Center [13]. This gene contains an aberrant splicing site in plants at the 5' part, which is the AGGTATTG sequence [17]. However, all pH-sensitive mutations exist in the downstream of the AGGTATTG sequence. The upstream before the AGGTATTG sequence was PCRRed by primes proe17-sGFP-F/sGFP-ph R394 from *sGFP* gene [9], which was modified to be soluble in plants, was fused to fragment PCRRed from Ratiometric pHluorin gene by primers sgfpPH394F/ NosEcor1R. This is named modified pHluorin gene. The transit peptide of proe17 from pea PCRRed by primers Proe17Xma1F/ proe17-sGFP-R was fused to the 5' end of the modified pHluorin gene by overlap PCR. The cassette was inserted into the plasmid PBI121 after the *CaMV35S* promoter region. The resulting construct was transformed to wild-type *A. thaliana* ecotype Columbia (Col-0) plants using *Agrobacterium tumefaciens* EHA105 mediated method [18].

2.2 Chloroplast fraction and protein extraction

Intact chloroplasts were isolated from one week old, two weeks old, four weeks old plants growing on MS medium followed the protocol previous reported [19]. After osmotic shock for 5 min on ice in LS buffer (10mM MES, 5mM MgCl₂, pH6.5), the thylakoids were pelleted at 12000 g for 5 min at 4°C. Thylakoids were then incubated in IB buffer (0.33M sorbitol, 50mM HEPS, pH8) with 0.1% Triton X-100 for 3 minutes on ice in order to release luminal proteins following the method previously reported (Hashimoto et al., 1996). After centrifuge at 144000g for 15 min, the supernatant (luminal protein) was precipitated by adding same amount of 30%TCA (Trichloroacetic acid) on ice for 15 minutes, followed by full speed centrifuge for 20 minutes. The pellet was resuspended in buffer (0.33M sorbitol, 16.7mM MES, 16.7mM tricine) PHed from 5.5 to 8.5 to the final concentration as 40µg/ml, seperately. Therefore, we had a series of luminal protein in different PH in hand for measuring the fluorescence intensity.

2.3 Quantification of GFP fluorescence

Fluorescence measurements were performed on a Fluorolog-3 spectrofluorometer (Jobin Yvon Inc., Edison, NJ). For Figure 3, the intact chloroplasts isolated from transgenic plants were fractioned to get luminal proteins as we described above in order to get the fluorescence intensity

1 corresponding to PH as control. Emission wavelength for the excitation spectra was at 530 nm.
2 For Figure 4, the intact chloroplasts isolated from transgenic plants were in IB and set to the final
3 concentration as 40 µg chlorophyll/ml. The sample was excited by 395 nm and 475 nm separately
4 for a period of 90 seconds. From 10 to 60 seconds the acidic light was on for activating
5 photosynthesis resulting in proton gradient.

6 7 **2.4 Cell Imaging**

8 For Figure 1, the isolated thylakoid images were observed using a Zeiss (PG-HITEC, Mem
9 Martins, Portugal) Axioskop 2 plus microscope equipped with a EBQ100 isolated fluorescent
10 lamp. For Figure 2, the in vitro chloroplast images were taken on a confocal laser scanning
11 microscope system (FV-1000; Olympus, Hamburg, Germany).

12 13 **3. Results**

14 **3.1 Expression pattern of ratiometric pHluorin in the transgenic Arabidopsis**

15 We transformed *A. thaliana* with a modified pH-sensitive GFP variant (ratiometric pHluorin)
16 targeted to the thylakoid lumen with the PrOE17 transit peptide. First we convinced the location of
17 the ratiometric pHluorin by microscope images. As shown in Figure 1, the targeted pHluorin is
18 indeed present in the thylakoid by microscope images of isolated thylakoids. Interestingly, we
19 found that the fluorescence signal from chloroplast-targeted pHluorins are the strongest in younger
20 leaves, and tend to be diminished in older leaves (Figure 1). In one week old plants, all of cells
21 showed strong fluorescence signal in the thylakoids (Figure 1C). In two weeks old plants, most of
22 the thylakoids still showed strong fluorescence signal (Figure 1G). In four weeks old plants, only
23 small portion of the thylakoids showed strong fluorescence signal (Figure 1J). Only 6.3%
24 thylakoids showed GFP signal by counting the thylakoids with and without fluorescence (Figure
25 1J). This result indicated that change of ratiometric pHluorin signal in thylakoid is associated with
26 the leaf development.

27 28 **3.2 Distribution of ratiometric pHluorin was inhomogeneous in leaves**

1 In order to know the distribution of ratiometric pHluorin in the transgenic Arabidopsis, the GFP
2 signal was investigated using intact leaves of one week old by confocal microscope (Figure 2).
3 RB-GFP was made by fusing Rubisco transit peptide with GFP, aiming to target GFP signal to
4 chloroplast stroma. Transgenic plants containing RB-GFP were used as control in chloroplasts
5 (Figure 2G). It clearly displayed that the chloroplasts in the guard cells had strong green
6 fluorescence (Figure 2A). However, the signal of the chloroplasts in the mesophyll cells was
7 inhomogeneous and only part of chloroplasts had green fluorescence. This small portion of
8 chloroplasts had strong green fluorescence but weak chlorophyll autofluorescence (Figure 1D to
9 F). Since chlorophyll autofluorescence indicated the density of chlorophyll and thylakoid
10 development, this result suggests the expression of ratiometric pHluorin was associated with the
11 density of chlorophyll and thylakoid development. Therefore our next question is whether
12 ratiometric pHluorin in transgenic plants has original ratiometric properties.

14 **3.3 In vivo properties of pHluorin**

15 In order to explore in vivo properties of ratiometric pHluorin in transgenic plants, we liberated
16 ratiometric pHluorin from the lumen of one week old *A. thaliana* thylakoids, and demonstrate that
17 this protein was indeed free in the soluble phase of the lumen and retains its pH-sensitive
18 properties as shown in Figure 3A. The released proteins were titrated in different pH buffer from
19 5.5 to 8.5 and the fluorescence intensity was measured by fluorometer. Excitation spectra of
20 ratiometric pHluorin were taken at emission wavelength 530nm for each pHed luminal protein.
21 The fluorescence intensity changed in a way of pH-dependent. The fluorescence intensity in the
22 left excitation peak at 395nm decreased, while in the right peak at 475nm increased during the pH
23 decreased. A calibration curve based on the ratio of fluorescence intensities from pHluorin excited
24 at 395 and 475 nm was shown in Figure 3B. It can be seen that this probe was indeed responsive
25 in the range required to examine the pH changes in lumen.

26 The chloroplasts from transgenic *A. thaliana* with lumen-targeted ratiometric pHluorin were
27 isolated and used to perform in vivo fluorometer assays. From 10 to 60 seconds the red light was
28 on for activating photosynthesis resulting in proton gradient. As Figure 4 showed, light-induced

1 changes were shown when excited in the peaks 395nm and 475nm, and both curves were
 2 deflected in the direction of acidification of the lumen. It is clear from these experiments that the
 3 stable transgenic plants with a pHluorin targeted to the lumen was successful to report bulk pH
 4 changes in response to actinic illumination.

5
 6 Figure 1. Ratiometric pHluorin signal change was associated with the leaf development. A to D, one week old
 7 seedling; E to H, two weeks old seedling; H to K, four weeks old seedling. A, B, E, F, H, I, Fluorescence
 8 microscope images for the leaves at different stage. C, D, G, H, J, K, Fluorescence microscope images for the
 9 isolated thylakoids at different stage. The images were taken by a Zeiss (PG-HITEC, Mem Martins, Portugal)
 10 Axioskop 2 plus microscope equipped with a EBQ100 isolated fluorescent lamp.

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Figure 2. Distribution of ratiometric pHluorin was inhomogeneous in transgenic *A. thaliana* leaves. A to C, ratiometric pHluorin in the guard cells. D to F, ratiometric pHluorin in the mesophyll cells. G to I, RB-GFP in the mesophyll cells as control. The images were taken on a confocal laser scanning microscope system (FV-1000; Olympus, Hamburg, Germany).

Figure 3. Ratiometric pHluorin proteins released from thylakoid is PH dependent. Excitation spectra of ratiometric pHluorin at emission wavelength 530nm, was taken at different pHs range from 5.5 to 8.5. Samples were proteins released from the thylakoid in transgenic Arabidopsis at two weeks old. Fluorescence measurements were performed on a Fluorolog-3 spectrofluorometer (Jobin Yvon Inc., Edison, NJ).

Figure 4. The fluorescence intensity of ratiometric pHluorin in isolated chloroplasts responded to illumination in a manner consistent with light-dependent lumen acidification. The samples were in import buffer at PH8.0, and excited by 395nm and 475 nm separately. From 10 to 60 seconds the red light was on for activating photosynthesis resulting in proton gradient.

4. Discussion

In the present work we have examined the thylakoid translocation of a modified pH-sensitive GFP named ratiometric pHluorin, which has double excitation wavelength at 395nm and 475nm,

1 displaying a reversible excitation ratio shift between pH 8.5 and 5.5. The ratiometric pHluorin was
2 fused to transit peptides derived from a nucleus encoded chloroplast precursor protein proe17. Our
3 analyses showed that the ratiometric pHluorin is transported into the thylakoid lumen. However,
4 efficient thylakoid transport can only be achieved in the guard cells, which means all of the
5 chloroplasts involved in the guard cells showed fluorescence signal (Figure 2A). While In the
6 mesophyll cells, only a small portion of chloroplasts gave fluorescence signal. This small portion
7 of chloroplasts had strong green fluorescence but weak chlorophyll autofluorescence (Figure 2),
8 which drove us think mature chloroplasts had no fluorescent proteins.

9 What is the reason for this phenomenon? Guard cells have much less chloroplasts compared
10 to mesophyll cells [20]. In most species studied, guard cells contain chloroplasts, which vary in
11 number depending upon the species [21]. Most species typically contain 10–15 chloroplasts per
12 guard cell, compared with 30–70 in a palisade mesophyll cell. Guard cell chloroplasts are often
13 smaller, with less thylakoid membrane stacks, and some are less well developed than those in
14 mesophyll cells, although these features vary across plant families. Considering the biological
15 properties of the guard cells and the mature chloroplasts, it appears reasonable to assume that the
16 ratiometric pHluorin proteins were digested in the lumen or did not fold in the right formation in
17 the mature chloroplasts.

18 Further experiments of calibration for ratiometric pHluorin proteins released from one week
19 old transgenic plants showed this protein was free in the soluble phase of the lumen and retained
20 its pH-sensitive properties (Figure 3). Also, fluorometer assay of isolated chloroplasts from
21 transgenic plants could report bulk pH changes in response to actinic illumination (Figure 4). We
22 assumed that the chloroplasts giving green fluorescence signal carried out photosynthesis
23 normally, and could be useful to the localization proton study for investigating the fundamental
24 questions about whether the protonmotive force (pmf) arose from protons delocalized throughout
25 the bulk aqueous volume or from protons localized at the membrane surface.

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