Title

A new fluorescence-based method to monitor the pH in the thylakoid lumen using GFP variants

Journal

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, 486(1)

ISSN

0006-291X

Authors

Yang, Hong

Pu, Xiaojun

Wang, Lu

et al.

Publication Date

2017

DOI

10.1016/j.bbrc.2016.12.032

Peer reviewed

Biochemical and Biophysical Research Communications 486 (2017) 1–5

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

A new fluorescence-based method to monitor the pH in the thylakoid lumen using GFP variants

Hong Yang [a], Xiaojun Pu [a], Lu Wang [a], Li Liu [a, b, **], Steven M. Theg [b, *]

[a] Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, 650201, China
[b] Department of Plant Biology, University of California – Davis, Davis, CA 95616, USA

ARTICLE INFO

Article history:
Received 2 December 2016
Accepted 5 December 2016
Available online 8 December 2016

Keywords:
Chloroplast
GFP
Genetically encoded biosensor
Thylakoid lumen pH
Proton dynamics

ABSTRACT

The ΔpH-dependent/Tat pathway is unique for using only the proton motive force for driving proteins transport across the thylakoid membrane in chloroplasts. 9-aminoacridine fluorescence quenching is widely used to monitor the ΔpH developed across the thylakoid membrane in the light. However, this method suffers from limited sensitivity to low ΔpH 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 ΔpH on ATP synthesis and protein transport.

1. Introduction

Chloroplasts are prominent organelles in green plants that are responsible for the reactions of photosynthesis through which most of the planet's reduced carbon and atmospheric O_2 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 questioned 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 are imported into the chloroplast via the Toc and Tic complex [4]. Stromal chaperones coordinate the delivery of energy to this process, and the stromal processing peptidase cleaves the transit peptide from the incoming precursor protein driven. From the stroma, lumen-located proteins continue across the thylakoid membrane process via four different pathways: the SRP, Sec, Tat and spontaneous pathways [4–6]. Precursors are recognized by their specific transport machineries through transit peptides. Not only native proteins but also foreign proteins fused to transit peptides can be directed to the organelle. It was reported that targeting of EGFP within chloroplasts by the cpTat pathway was successful [7].

Wild type GFP cDNA from *Aequorea victoria* has been modified for use in plants by optimizing codon usage and removal of cryptic introns to achieve higher signal intensity, improved solubility, lower temperature sensitivity and different spectral characteristics [8]. Engineered Av-GFP is now in widespread use in plants to study protein localization, hormone movement, protein-protein interaction, gene expression, and cellular and extracellular ion dynamics [9–12]. With the development of pH-indicating pHluorins [13], it has become possible to measure proton activity and cytoplasmic

* Corresponding author.
** Corresponding author.
E-mail addresses: liulia@mail.kib.ac.cn (L. Liu), smtheg@ucdavis.edu (S.M. Theg).

http://dx.doi.org/10.1016/j.bbrc.2016.12.032
0006-291X/

free Ca^{2+} concentrations in roots, pollen or the cytoplasm under stress [8,14,15]. Ratiometric pHluorin is a pH-sensitive variant of Av-GFP, containing eight amino acid mutations (E132D, S147E, N149L, N164I, K166Q, I167V, R168H and L220F). The protein can be excited at 395 nm and 475 nm, and displays a reversible excitation ratio shift between pH 7.5 and 5.5 [13].

Previous measurements relating the ΔpH component of the pmf to protein transport on the cpTat pathway were made using 9-aminoacridine (9-AA) fluorescence as readout of the ΔpH. 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 ΔpH, we constructed a novel pH-sensitive GFP variant by overlapping the first 1–395 bp of sGFP (modified as described above for increased solubility) with the last 324 bp of ratiometric pHluorin containing all of the pH sensitivity-mutated amino acids together to get a new soluble and high quantum efficiency fluorescent protein. This modified ratiometric pHluorin was then driven by the 35S promoter and targeted to the chloroplast lumen by fusing it to the prOE17 transit peptide before its cDNA. A series of experiments were performed to characterize the phenotype of the transformed *A. thaliana* and to measure the proton gradient by lumen-localized pHluorin. Fluorescence images showed that pHluorin indeed localized to the thylakoid lumen. Protein recovered from this compartment retained its pH sensitivity. Confocal imaging was applied to mesophyll and guard cells of transformants, and showed changes of the fluorescence signal responding to photosynthetic acidity. These experiments demonstrate the possibilities of applying this novel approach to measure the light-dependent acidification of the lumen. We also discussed the potential problems that may arise when using this probe of the lumen pH.

2. Materials and methods

2.1. Plasmid constructs and transformation

The ratiometric pHluorin gene was provided by Dr. G. Miesenbock of Oxford University [13]. This gene contains an aberrant splicing site utilized in plants in the 5′ region at the sequence AGGTATTG [17]. Fortuitously, all pH sensitivity-conferring mutations exist downstream of this region. The upstream region before the AGGTATTG sequence was PCR amplified by primers proe17-sGFP-F/sGFP-ph R394 from the *sGFP* gene [9], which was modified to be soluble in plants. This product was amplified from the Ratiometric pHluorin gene by primers sgfpPH394F/NosEcor1R. We refer to this hereafter as the modified pHluorin gene. The transit peptide cDNA of prOE17 from pea amplified by primers Proe17Xma1F/proe17-sGFP-R, was fused to the 5′ end of the modified pHluorin gene by overlap PCR. The primers are listed in Supplemental Table 1. The resulting cassette was inserted into the plasmid PBI121 following the *CaMV35S* promoter. The resulting construct was transformed into wild-type *A. thaliana* ecotype Columbia (Col-0) plants using the *Agrobacterium tumefaciens* EHA105 mediated method [18].

2.2. Chloroplast fraction and protein extraction

Intact chloroplasts were isolated from one, two, or four weeks old plants growing on MS medium following the protocol previous reported in Ref. [19]. For isolation of thylakoid lumen proteins, intact chloroplasts were osmotically shocked for 5 min on ice in LS buffer (10 mM MES, 5 mM $MgCl_2$, pH6.5) and the thylakoids were pelleted at 12,000×*g* for 5 min at 4 °C. Thylakoids were then incubated in IB buffer (0.33 M sorbitol, 50 mM HEPS, pH8) with 0.1% Triton X-100 for 3 min on ice in order to release lumenal proteins following the method previously reported (Hashimoto et al., 1996). After centrifugation at 144,000×*g* for 15 min, the supernatant (lumenal proteins) was divided into different aliquots, each of which was precipitated by adding an equal volume of 30% TCA on ice for 15 min, followed by centrifugation at 12,000×*g* for 20 min. The pellets were resuspended in buffer (0.33 M sorbitol, 16.7 mM MES, 16.7 mM Tricine and 16.7 mM PIPES) with the pH set from 5.5 to 8.5. The final protein concentration in the resuspensions was set to 40 μg/ml.

2.3. Quantification of GFP fluorescence

Fluorescence measurements were performed on a Fluorolog 3–22 spectrofluorometer (Horiba Scientific, Edison, NJ). For Fig. 3, the intact chloroplasts isolated from transgenic plants were fractioned to obtain lumenal proteins as described above in order to measure the fluorescence intensity as a function of pH. The emission wavelength for the excitation spectra was at 530 nm. For Fig. 4, the intact chloroplasts isolated from transgenic plants were in IB at a final concentration of 40 μg chlorophyll/ml. Fluorescence was excited at 395 nm and 475 nm separately for a period of 90 s. From 10 to 60 s the samples were illuminated with red actinic light, which activated the photosynthetic electron transport chain and resulted in establishment of a proton gradient.

2.4. Cell imaging

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

3. Results

3.1. Expression pattern of ratiometric pHluorin in the transgenic Arabidopsis

We transformed *A. thaliana* with a modified pH-sensitive GFP variant (ratiometric pHluorin) targeted to the thylakoid lumen with the prOE17 transit peptide. First we identified the location of the ratiometric pHluorin by microscopy. As shown in Fig. 1, the targeted pHluorin was indeed present in the thylakoids as seen in microscopic images of isolated thylakoids. Interestingly, we found that the fluorescence signal from chloroplast-targeted pHluorins are the strongest in younger leaves, and tend to be diminished in older leaves (Fig. 1). In one week old plants, all of cells showed strong fluorescence signals in the thylakoids (Fig. 1C). After two weeks most of the thylakoids still displayed a strong fluorescence signal (Fig. 1G), but after four weeks only a small portion of the thylakoids showed a strong fluorescence signal (Fig. 1J). Counting of thylakoids with and without GFP fluorescence revealed that only 6.3% thylakoids retained the GFP signal through four weeks (Fig. 1 J, K). This result indicated that the ratiometric pHluorin signal in thylakoids is associated with the leaf development.

3.2. Distribution of ratiometric pHluorin was inhomogeneous in leaves

In order to more accurately discern the distribution of ratiometric pHluorin in the transgenic Arabidopsis, the GFP signal was investigated in one week old intact leaves using a confocal microscope (Fig. 2). RB-GFP was made by fusing the Rubisco small

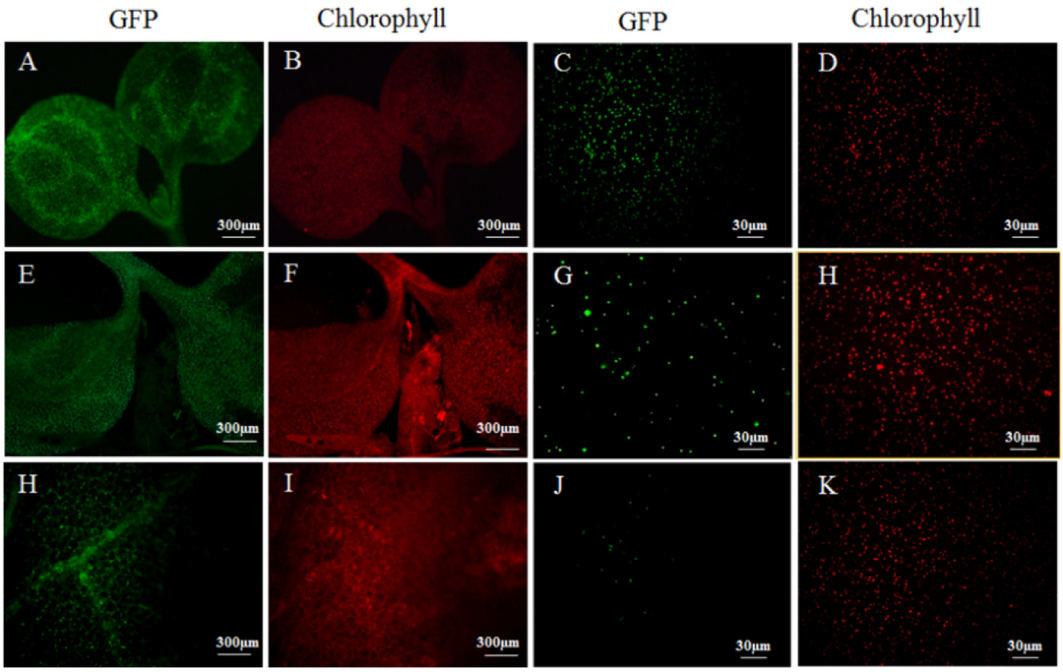

Fig. 1. Ratiometric pHluorin signal in the lumen was associated with the leaf development. A to D, one week old seedling; E to H, two weeks old seedling; H to K, four weeks old seedling. A, B, E, F, H, I, Fluorescence microscope images for the leaves at different ages. C, D,G, H, J, K, Fluorescence microscope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

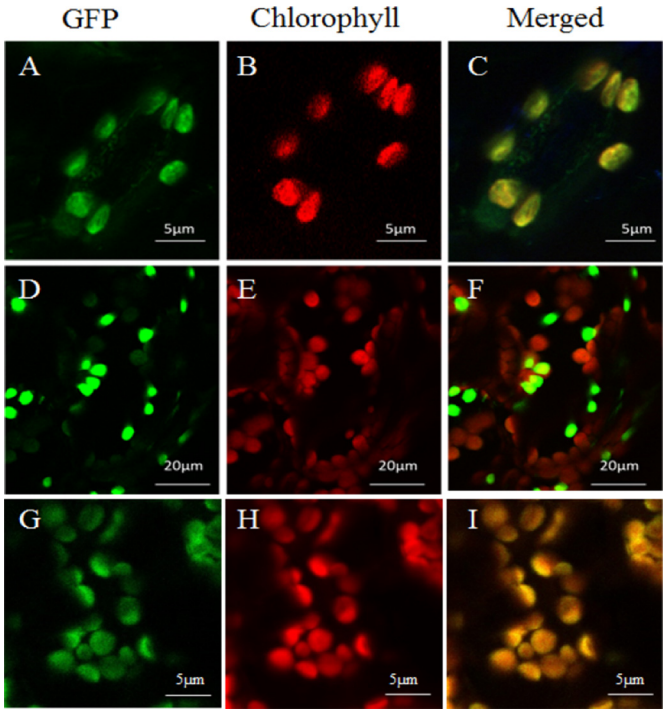

Fig. 2. Distribution of ratiometric pHluorin was inhomogeneous in transgenic *A. thaliana* leaves. A to C, ratiometric pHluorin in the guard cells. D to F, ratiometric pHluorin in the mesophyll cells. G to I, RB-GFP in the mesophyll cells as control. The images were taken on a confocal laser scanning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

subunit transit peptide with GFP, targeting GFP to the chloroplast stroma. Transgenic plants containing RB-GFP were used to stably label the chloroplasts (Fig. 2G). Fig. 2A clearly shows that the chloroplasts in the guard cells displayed a uniform and robust green fluorescence signal. However, the signal from the chloroplasts in the mesophyll cells was inhomogeneous and only some of the chloroplasts exhibited green fluorescence. This small percentage of chloroplasts had strong green fluorescence but weak chlorophyll autofluorescence (Fig. 2D–F). Since chlorophyll autofluorescence indicated the density of chlorophyll and thus thylakoid development, this result suggests the expression of ratiometric pHluorin was strongly influenced by the state of thylakoid development.

3.3. *In vivo properties of pHluorin*

In order to explore in vivo properties of ratiometric pHluorin in transgenic plants, we released ratiometric pHluorin from the lumen of one week old *A. thaliana* thylakoids by mild detergent treatment. This demonstrated that the protein was indeed free in the soluble phase of the lumen. The released protein was placed in buffer ranging from pH 5.5 to 8.5 and the fluorescence intensity was measured. Excitation spectra of ratiometric pHluorin were recorded at an emission wavelength of 530 nm for each tested pH. As expected, the fluorescence intensity in the excitation peak at 395 nm decreased as the pH decreased, while the peak at 475 nm increased. A calibration curve based on the ratio of fluorescence intensities from pHluorin excited at 395 and 475 nm is shown in Fig. 3B. It can be seen that this probe was indeed responsive to pH in the range required to examine light-dependent pH changes in lumen.

The chloroplasts from transgenic *A. thaliana* with lumen-targeted ratiometric pHluorin were isolated and used to monitor light-dependent fluorescent changes in the thylakoid lumen. Actinic red light was used to activate photosynthetic electron transport from 10 to 60 s, resulting in proton deposition into the lumen. As shown in Fig. 4, light-induced fluorescence changes were observed when pHluorin was excited at 395 nm and 475 nm, and both curves showed deflections in the direction of acidification of the lumen. It is clear from these experiments that the stable transgenic plants with a pHluorin targeted to the lumen

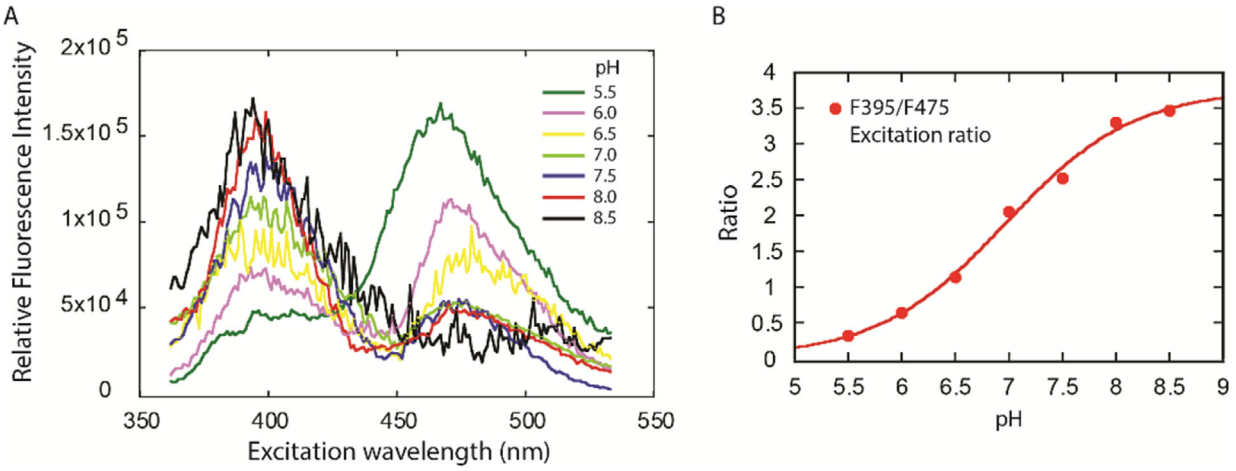

Fig. 3. Fluorescence of ratiometric pHluorin proteins released from thylakoids is pH dependent. Excitation spectra of ratiometric pHluorin at emission wavelength 530 nm, was taken at different pH values from 5.5 to 8.5. Samples were proteins released from thylakoids in transgenic Arabidopsis at two weeks old. Fluorescence measurements were performed in a Fluorolog-322 spectrofluorometer (Horiba Scientific, Edison, NJ).

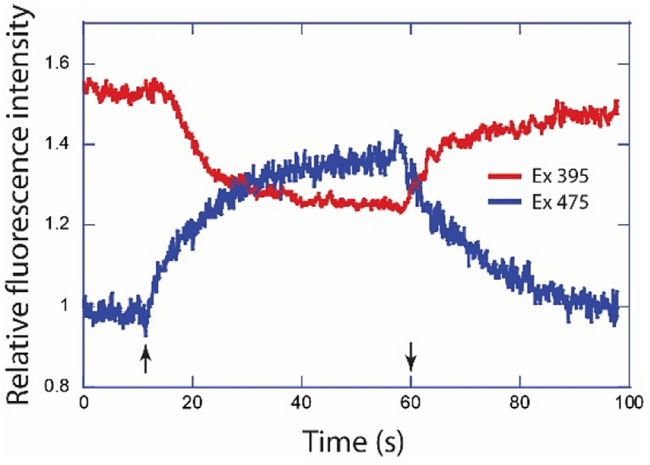

Fig. 4. The fluorescence intensity of ratiometric pHluorin in isolated chloroplasts responded to illumination in a manner consistent with light-dependent lumen acidification. The samples were in import buffer at pH 8.0, and fluorescence was excited at 395 nm and 475 nm as indicated; emission was monitored at 530 nm. Upward (on) and downward (off) arrows indicate illumination with red actinic light.

successfully reported bulk pH changes in response to actinic illumination.

4. Discussion

In the present work we have examined the thylakoid translocation of a modified pH-sensitive GFP referred to as ratiometric pHluorin [13], which displays a pH-dependent excitation spectrum between pH 8.5 and 5.5. The ratiometric pHluorin was fused to a transit peptide derived from the nucleus-encoded chloroplast precursor protein prOE17. Our analyses showed that the ratiometric pHluorin is transported into the thylakoid lumen. However, efficient thylakoid transport was only observed in guard cells, wherein all chloroplasts displayed a fluorescence signal (Fig. 2A). In contrast, only a small portion of the chloroplasts in mesophyll cells showed pHluorin fluorescence. This small portion of the chloroplasts displayed strong green fluorescence but weak chlorophyll autofluorescence (Fig. 2), suggesting that the fluorescent protein was missing from the mature plastids.

What is the reason for this phenomenon? In most plant species studied, guard cells contain chloroplasts but they are fewer in number than in mesophyll cells [20,21]. Most species typically contain 10–15 chloroplasts per guard cell, compared with 30–70 in a palisade mesophyll cell. Guard cell chloroplasts are often smaller than their mesophyll cell counterparts, with fewer thylakoid membrane stacks, and are perhaps less well developed, although these features vary across plant families. Considering the housekeeping properties of chloroplasts, it appears reasonable to assume that many of the ratiometric pHluorin proteins were digested in the lumen or did not fold into their correct conformation in the mature chloroplasts.

Further experiments demonstrated that ratiometric pHluorin proteins were free in the soluble phase of the lumen and retained their pH-sensitive properties (Fig. 3). Also, fluorescence assays with isolated chloroplasts from transgenic plants could, in some instances, report pH changes in the lumen in response to actinic illumination (Fig. 4). We assumed that the chloroplasts showing the green fluorescence signal carried out photosynthesis normally, although this point remains to be tested vigorously. Future experiments will be aimed at determining conditions to better maintain the pHluorin signals in mature plants, and will explore the utility of this new probe of the lumen pH under conditions where previous techniques have proved difficult or unreliable.

Acknowledgments

The constructs were created in our lab by modifying pHluorins, generously provided by Dr. G. Miesenbock of Oxford University, for use in plants. This work was supported by the Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences of the U.S. Department of Energy through Grant DE-FG02-03ER15405 and by the 100 Talents Program of The Chinese Academy of Sciences and the National Natural Science Foundation of China (31571262).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.12.032.

References

[1] P. Mitchell, Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. 1966, Biochimica Biophysica Acta 1807 (2011) 1507–1538.
[2] P. Mitchell, Chemiosmotic coupling in oxidative and photosynthetic phosphorylation, Biol. Rev. Camb Philos. Soc. 41 (1966) 445–502.
[3] R.A. Dilley, On why thylakoids energize ATP formation using either delocalized or localized proton gradients - a Ca2+ mediated role in thylakoid stress responses, Photosynth. Res. 80 (2004) 245–263.
[4] H.M. Li, C.C. Chiu, Protein transport into chloroplasts, Annu. Rev. Plant Biol. 61 (2010) 157–180.
[5] P. Jarvis, C. Robinson, Mechanisms of protein import and routing in chloroplasts, Curr. Biol. 14 (2004) R1064–R1077.
[6] P. Jarvis, Targeting of nucleus-encoded proteins to chloroplasts in plants, New Phytol. 179 (2008) 257–285.
[7] J.P. Marques, M.H. Schattat, G. Hause, I. Dudeck, R.B. Klösgen, In vivo transport of folded EGFP by the ΔpH/TAT-dependent pathway in chloroplasts of Arabidopsis thaliana, J. Exp. Bot. 55 (2004) 1697–1706.
[8] N. Moseyko, L.J. Feldman, Expression of pH-sensitive green fluorescent protein in Arabidopsis thaliana, Plant Cell Environ. 24 (2001) 557–563.
[9] W.L. Chiu, Y. Niwa, W. Zeng, T. Hirano, H. Kobayashi, J. Sheen, Engineered GFP as a vital reporter in plants, Curr. Biol. 6 (1996) 325–330.
[10] R. Waadt, P.K. Hsu, J.I. Schroeder, Abscisic acid and other plant hormones: methods to visualize distribution and signaling, Bioessays 37 (2015) 1338–1349.
[11] S. Okumoto, A. Jones, W.B. Frommer, Quantitative imaging with fluorescent biosensors, Annu. Rev. Plant Biol. 63 (2012) 663–706.
[12] D.M. Chudakov, S. Lukyanov, K.A. Lukyanov, Fluorescent proteins as a toolkit for in vivo imaging, Trends Biotechnol. 23 (2005) 605–613.
[13] G. Miesenbock, D.A. De Angelis, J.E. Rothman, Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins, Nature 394 (1998) 192–195.
[14] D. Gao, M.R. Knight, A.J. Trewavas, B. Sattelmacher, C. Plieth, Self-reporting Arabidopsis expressing pH and [Ca2+] indicators unveil ion dynamics in the cytoplasm and in the apoplast under abiotic stress, Plant Physiol. 134 (2004) 898–908.
[15] S.J. Swanson, W.G. Choi, A. Chanoca, S. Gilroy, In vivo imaging of Ca2+, pH, and reactive oxygen species using fluorescent probes in plants, Annu. Rev. Plant Biol. 62 (2011) 273–297.
[16] S.M. Theg, C. Tom, Measurement of the ΔpH and electric field developed across Arabidopsis thylakoids in the light, Chloroplast Res. Arabidopsis Methods Protoc. II (2011) 327–341.
[17] J. Haseloff, K.R. Siemering, D.C. Prasher, S. Hodge, Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly, Proc. Natl. Acad. Sci. 94 (1997) 2122–2127.
[18] X. Zhang, R. Henriques, S.S. Lin, Q.W. Niu, N.H. Chua, Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method, Nat. Protoc. 1 (2006) 641–646.
[19] P.H. Su, H.M. Li, Stromal Hsp70 is important for protein translocation into pea and Arabidopsis chloroplasts, Plant Cell 22 (2010) 1516–1531.
[20] T. Lawson, Guard cell photosynthesis and stomatal function, New Phytol. 181 (2009) 13–34.
[21] T. Lawson, K. Oxborough, J.I. Morison, N.R. Baker, The responses of guard and mesophyll cell photosynthesis to CO_2, O_2, light, and water stress in a range of species are similar,, J. Exp. Bot. 54 (2003) 1743–1752.