RESEARCH PAPER

Dynamic imaging of glucose flux impedance using FRET sensors in wild-type Arabidopsis plants

Bhavna Chaudhuri*, Friederike Hörmann*,† and Wolf B. Frommer‡

Department of Plant Biology, Carnegie Institution for Science, 260 Panama Street, Stanford, CA 94305, USA

* These authors contributed equally to this work.
† Present address: Mikrobiologie, University Hohenheim, Garbenstr. 30, D-70593 Stuttgart, Germany.
‡ To whom correspondence should be addressed: E-mail: wfrommer@stanford.edu

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Abstract

Quantitative and dynamic analysis of metabolites and signalling molecules is limited by technical challenges in obtaining temporally resolved information at the cellular and compartmental level. Real-time information on signalling and metabolite levels with subcellular granularity can be obtained with the help of genetically encoded FRET (Förster resonance energy transfer) nanosensors. FRET nanosensors represent powerful tools for gene discovery, and analysis of regulatory networks, for example by screening mutants. However, RNA silencing has impaired our ability to express FRET nanosensors functionally in Arabidopsis plants. This drawback was overcome here by expressing the nanosensors in RNA silencing mutants. However, the use of silencing mutants requires the generation of homozygous lines deficient in RNA silencing as well as the mutation of interest and co-expression of the nanosensor. Here it is shown that dynamic changes in cytosolic glucose levels can readily be quantified in wild-type Arabidopsis plants at early stages of development (7–15 d) before silencing had a major effect on fluorescence intensity. A detailed protocol for screening 10–20 mutant seedlings per day is provided. The detailed imaging protocol provided here is suitable for analysing sugar flux in young wild-type plants as well as mutants affected in sugar signalling, metabolism, or transport using a wide spectrum of FRET nanosensors.

Key words: Energy transfer, fluorescence resonance, flux, glucose, nanosensor, root tip.

Introduction

Quantitative and dynamic metabolite analysis with cellular and subcellular granularity is crucial for the understanding of metabolic networks, especially in the context of flux regulation. Isotope tracers are effective tools for determining pathway structure, for example in unravelling the photosynthetic dark reactions (Aronoff et al., 1947). Radio-tracers are still a primary tool for the characterization of cellular transport processes (Chen et al., 2010). Stable isotope techniques are applied to obtain comprehensive flux analysis with the aim of producing system-wide flux maps of metabolic networks (Wiechert et al., 2007). Complementary to isotope-based flux methods, imaging-based metabolite analysis with genetically encoded nanosensors based on the principle of Förster resonance energy transfer (FRET; Förster, 1948) measures dynamic changes in metabolite concentration with cellular and subcellular resolution (Okumoto et al., 2008). Typically, FRET nanosensors consist of a recognition element (in the case of metabolites, typically a member of the bacterial periplasmic-binding protein family) fused to a reporter element (a fluorophore pair having overlapping emission spectra) (Okumoto et al., 2008; Frommer et al., 2009). Binding of the ligand (metabolite of interest) leads to a conformational change of the recognition element, triggering a change in the energy transfer between the fluorophores. The FRET change directly correlates with the analyte concentration and follows a binding isotherm with a detection range of approximately plus or minus one order of magnitude around the binding...
constant of the recognition element of the nanosensor. Similar nanosensors have been developed for many signalling molecules such as calcium; a review of the corresponding literature goes beyond the scope of this introduction (see, for example, Frommer et al., 2009).

FRET nanosensors have successfully been deployed in yeast to monitor glucose influx and to analyse transport mutants (Bermejo et al., 2010). To be able to cover a range of concentrations from tens of nanomolar to tens of millimolar, a series of affinity mutants were generated (Fehr et al., 2003; Deuschle et al., 2005, 2006; Bermejo et al., 2010). These affinity mutants also serve as internal controls and exclude artefacts caused by other parameters on the nanosensors such as pH or ionic conditions. In human cells, the nanosensors were used to monitor glucose transport across the nuclear and endoplasmic reticulum membranes (Fehr et al., 2004, 2005; Takanaga et al., 2008; Takanaga and Frommer, 2010) or glutamate eflux from hippocampal neurons in response to electrical stimulation (Okumoto et al., 2005; Hires et al., 2008). In plants, the FRET sugar sensors have predominantly been used to monitor relative changes of the steady-state sugar levels within a subcellular compartment in response to perfusion of the cells or tissues with modulated square pulses of FRET glucose and sucrose sensors (Chaudhuri et al., 2008). The use of square pulses is similar to what is used for impedance measurements in electrical engineering, where the shape of output waves provides information on system properties [dynamic imaging of flux impedance (DIFI); Fig. 1]. Similar to electrical impedance measurements, low conductivity leads to low or undetectable sensor responses, as in the case of the phosphate sensor FLIPPi in Chinese hamster ovary (CHO) cells (Gu et al., 2006), or the glucose sensor in HEK293T cells (Takanaga and Frommer, 2010). Low conductivity can be overcome by overexpressing transporters in the respective cell lines (Gu et al., 2006; Takanaga and Frommer, 2010). Impedance measurements are thus suitable for gene discovery by co-expressing unknown membrane proteins with the sensors (Chen et al., 2010), or to study the regulation of transporters. It should be noted that the steady-state levels of the ions or metabolites depend not only on membrane conductivity but rather on the ratio of membrane conductivity and intracellular conversion compartmentation (Okumoto et al., 2008).

One of the initial difficulties in implementing FRET sensor technology in plants was that the nanosensors are subject to gene silencing in wild-type Arabidopsis plants (Deuschle et al., 2006). Gene silencing was overcome by expressing the nanosensors in mutant plants deficient in gene silencing (rdr6-11 and sgs3) (Peragine et al., 2004), allowing the monitoring of steady-state glucose or sucrose levels in the cytosol of epidermal leaf cells as well as intact roots of axenically or soil-grown plants (Deuschle et al., 2006; Chaudhuri et al., 2008). The analysis of glucose and sucrose flux impedance in roots tips in the presence of inhibitors indicated the presence of facilitative sugar uptake transporters of unknown nature (Chaudhuri et al., 2007, 2008). Similar DIFI impedance analyses using glutamine sensors in Arabidopsis roots also required the use of rdr6 silencing mutants (Yang et al., 2010).

Metabolite FRET nanosensors have successfully been used to characterize yeast glucose transport mutants (Bermejo et al., 2010). In plants, however, the analysis of mutants is more challenging since it requires the use of triple mutants, typically homozygous recessive single (or double) mutants in the gene of interest, combined with the homozygous recessive silencing mutant, and the plants have to co-express a FRET nanosensor.

Here it is shown that young wild-type Arabidopsis seedlings can be used to monitor glucose flux with the FRET nanosensors in root tips due to low efficacy of gene silencing in early development. Moreover, a detailed protocol for screening of Arabidopsis mutants for effects on sugar flux is provided.

Materials and methods

Plant material and FLIP constructs

Arabidopsis Col-0 transformants (T_{2}) expressing FLIPglu-170nΔ13 (line 6), FLIPglu-2μΔ13 (line 2), and FLIPglu-600μΔ13 (line 8) were used for the DIFI analyses. The plasmid constructs used here have been described previously (Deuschle et al., 2006).

Plant growth

Seedlings were germinated on hydroponic medium (1 mM KH_{2}PO_{4}, 1 mM MgSO_{4}, 0.25 mM K_{2}SO_{4}, 0.25 mM CuCl_{2}, 2 mM NH_{4}NO_{3}, 0.1 mM Na-Fe-EDTA, 50 μM KCl, 30 μM H_{3}BO_{3}, 5 μM MnSO_{4}, 1 μM ZnSO_{4}, 1 μM CuSO_{4}, 0.7 μM NaMoO_{4} pH 5.8 adjusted with KOH) (Loque et al., 2005) buffered with 20 mM MES (solidified with 0.7% agar and supplemented with 2% sucrose) and incubated in a growth chamber under a 16 h light/8 h dark regime at 50% humidity and 22 °C for 7 d. Seedlings were transferred to the same medium lacking the sucrose for 2–6 d prior to imaging, depending on the affinity of the nanosensor expressed in the plants.

FRET imaging

Seedlings grown on hydroponic media were mounted on coverslips (24×50 mm No. 1½, VWR) using medical adhesive (Stock No. 7730, Hollister) to restrict movement (Deuschle et al., 2006; Young et al., 2006). Chambers used for screening were made with plastic clay (Sculpey, www.sculpey.com) and were variable in size and volume (1–2 ml), thus preventing quantitative analysis of accumulation/elimination rates because of limiting perfusion kinetics (Figs 3A–E, 5). For qualitative analyses, the clay chamber was filled with hydroponic medium, perfusion tubing was mounted, and
roots were perfused at 3 ml min\(^{-1}\) (Fig. 3F, G). Ratio imaging was performed on an inverted fluorescence microscope (DM IRE2, Leica) with a QuantEM digital camera (Roper) and a \(\times 20\) oil objective (HC PL APO \(\times 20\)/0.74MM CORR, Leica, Germany). Dual emission intensities were recorded simultaneously using a DualView with dual CFP/YFP-ET filter sets [high transmission modified Magnetron sputter-coated filter sets ET470/24m (470 indicates the emission wavelength, /24 indicates bandwidth); ET535/3, Chroma, USA] and Slidebook software (Intelligent Imaging Innovations Inc., USA). The DualView (or similar image splitter from other companies) enables simultaneous recording of both emission wavelengths without mechanical filter switching. For most metabolic imaging studies, filter wheels that automatically switch between the two emission wavelengths are equally suitable. Software for FRET image acquisition is available from a variety of commercial vendors, as scripts from individual labs, or can be implemented using the free software package ImageJ (rsb.info.nih.gov/ij). Individual root tips were imaged while perfusing roots with glucose expected to saturate the sensor (20 mM) using a peristaltic pump (Fig. 3F). Seedlings that showed a FRET change in response to sugar addition were transferred to a commercial P1 chamber (Warner, USA; volume 600–700 µl) or an RC-26 chamber (Warner, USA; 400–500 µl). For younger seedlings, even smaller chambers can be used in order to minimize the possibility that uptake/efflux rates are limited by the perfusion system. The clay chamber system can be used to screen \(\sim 12\) plants within 3 h. Responsive seedlings can then be mounted in an RC-26 chamber and analysed with three sugar concentrations at 25, 50, and 100% saturation (25 min per experiment; 5–12 min per concentration), permitting the screening of 10–20 plants per day. Protocols for data analysis have been described (Takanaga et al., 2008).

Calculation of exchange rates for the RC-26 chamber

The exchange rate was calculated by using the same perfusion set-up that had been used for FRET imaging but using the fluorescent dye Alexa Fluor 430 (Invitrogen) and perfusion at a rate of 3 ml min\(^{-1}\). Exchange rates were determined by setting the maximum fluorescence intensity as 100% and measuring the time needed until 85% intensity was reached (15% for removal of dye) in the linear uptake and efflux phases, respectively.

Results and Discussion

Expression of the FRET glucose nanosensor in axenically grown wild-type seedlings

Expression of the FRET nanosensor is subject to gene silencing in wild-type Col-0 Arabidopsis plants (Fig. 2A, B). Silencing can be prevented by expressing FLIPv-600µµA13 series nanosensors in rdr6 mutants (Fig. 2C). Silencing is typically low during germination and builds up during further development (Schubert et al., 2004). To circumvent the use of silencing mutants, tests were carried out to determine whether young wild-type (Arabidopsis thaliana

FLIPv-600µµA13 is shown. (D) Comparison of YFP and CFP fluorescence intensity in wild-type and rdr6 transformants (average background-corrected YFP and CFP values for six independent wild-type and rdr6 root tips were calculated from the baseline values ±SE). (E) FRET ratio image of a root tip of an Arabidopsis wild-type seedling expressing FLIPv-600µµA13.
Col-0) seedlings express sufficient FRET sensor levels in roots. Previous work had found that most transformants in a wild-type background were silenced when analysing leaves of mature plants (Deuschle et al., 2006). To analyse root expression, here seedlings were grown axenically. Fluorescence levels of the FRET nanosensors were significantly lower compared with rdr6, but sufficiently high for analysis in seedlings with ages of up to ∼15 d (Fig. 2B–E). The average yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) fluorescence levels in wild-type plants were significantly lower at 825±46 A.U. and 428±23 A.U., respectively, compared with rdr6 transformants, in which the levels were ∼10× higher (8615±1232 A.U. for YFP and 3402±387 A.U. for CFP; Fig. 2D). Nanosensor expression was detected in roots even after the trans-gene had been silenced completely in the aerial parts (data not shown). For quantitative analysis of glucose levels described here, ∼10-day-old wild-type seedlings were used.

To screen seedlings rapidly, a simple polymer clay chamber system was developed. Transfer of a single seedling to a hand-made clay chamber and fixation on a coverslip using medical adhesive typically took 5 min, thus allowing the preparation of 10–12 seedlings within 1 h (Fig. 3A–E). Coverslips with single seedlings were then analysed for FRET changes in response to perfusion with a single pulse of a saturating concentration of glucose (Fig. 3F, G). Seedlings were first perfused with hydroponic medium lacking glucose until a stable baseline was reached, cells were then perfused for 2–5 min with glucose, and finally the glucose medium was replaced with glucose-free medium and analysis was completed after the FRET ratio had returned to the baseline (7–10 min). Complete analysis of a single seedling thus took ∼15 min. Up to 20 plants were screened for FRET changes in response to perfusion with glucose within a single day using the simple polymer clay chambers.

Coverslips with seedlings that yielded a measurable FRET change after perfusion with sugar were transferred to a laminar flow perfusion chamber (Fig. 3H) and analysed more carefully by applying modulated square pulses of glucose (DIFI; Figs 1, 4). This approach may be suitable for screening mutant or chemical libraries to identify regulators or novel drugs that affect sugar flux.

Limitation of response kinetics by perfusion kinetics

A prerequisite for rapid kinetic analyses, for example quantification of the accumulation and elimination rates (Fig. 4), is that perfusion rates exceed the accumulation/elimination rates. Adequate chamber size, laminar flow design, and the velocity of perfusion are important parameters for rapid kinetic analyses. Large volume chambers require high flow rate perfusion systems such as peristaltic pumps or pressurized systems to ensure that perfusion does not limit the response kinetics. In the RC-26 chamber (World Precision; liquid level ∼3 mm; volume ∼500 µl), and at a perfusion rate of 3 ml min⁻¹ it took ∼20 s to change 85% of the buffer and ∼22 s to remove 85% of the fluorescent dye from the chamber (Alexa Fluor 430; Fig. 5). Since FRET changes occurred at a similar time scale (Figs 4, 6), the RC-26 chamber may not be optimal for fast kinetic analysis. Smaller chambers such as the RC-24E (Warner, USA; volume at 3 mm liquid level 150 µl) may enable faster exchange. For smaller chambers, slower perfusion systems based on gravity are sufficient, reducing the risk of transmission of oscillations caused by the peristaltic perfusion (Okumoto et al., 2008; Takanaga and Frommer, 2010). Microfluidic devices that allow analyses of multiple roots in parallel might be a means for further increasing the throughput.

Response of the FRET glucose nanosensors in wild-type Arabidopsis roots

To reduce endogenous sugar levels before analysis, seedlings were initially grown on solidified hydroponics media lacking
glucose or sucrose (Deuschle et al., 2006; Chaudhuri et al., 2008). However, extended starvation led to low fluorescence levels, possibly due to reduced translation efficiency. Since absolute nanosensor expression levels were lower in wild-type seedlings compared with rdr6 transformants (Fig. 2D, E), it was important to identify the window in which fluorescence levels were sufficient for analysis while ensuring that endogenous sugar levels were below the detection level of the nanosensor. First, DIFI analysis of wild-type Arabidopsis transformants expressing FLIPglu-600L13 (Kd; 600 µM; range 60–6000 µM) was performed (Fig. 4). For FLIPglu-600µL13 seedlings, starvation for 2–3 d proved sufficient for depletion of endogenous glucose. Roots were perfused with modulated square pulses of glucose to determine the apparent in vivo K0.5. The apparent in vivo K0.5 for FLIPglu-600µL13 was ~0.8–1 mM (Fig. 4). Thus steady-state levels of glucose were ~75% of the external concentration. It took ~1 min before the nanosensor saturated and 2–3 min to return to the baseline after removal of glucose (Chaudhuri et al., 2008). The accumulation and elimination rates were in a similar range to the perfusion kinetics (especially if the potential effect of the presence of a root on laminar flow is taken into account).
Thus the actual accumulation and elimination rates are potentially equal or faster compared with those observed here. No apparent differences in terms of the maximal Δ ratios or in vivo $K_{0.5}$ were observed when compared with rdr6 plants expressing the nanosensors (Chaudhuri et al., 2008).

Similar analyses were performed for Arabidopsis Col-0 seedlings expressing FLIPglu-Δ13 affinity mutants FLIPglu-170nΔ13 ($K_d \sim 170$ nM; range $17–1700$ nM) or FLIPglu-2μΔ13 ($K_d \sim 2$ μM; range $0.2–20$ μM; Fig. 6A, B). Seedlings were grown on solidified hydroponic media supplemented with 2% sucrose to ensure high levels of nanosensor expression. Seedlings were transferred onto media lacking sucrose for 4–6 d before imaging. For plants expressing FLIPglu-170nΔ13 it took 5–6 d of sugar depletion before endogenous sugar levels had dropped below the detection level of the nanosensor, whereas for FLIPglu-2μΔ13-expressing seedlings 4–5 d of starvation were required. The apparent in vivo $K_{0.5}$ for FLIPglu-170nΔ13 was $\sim 1$ μM (Fig. 6A). Thus in this low concentration range, steady-state levels of glucose were only $\sim 20\%$ of the external concentration. For FLIPglu-2μΔ13 the apparent in vivo $K_{0.5}$ was $\sim 4$ μM (Fig. 6B). In this case, steady-state levels of glucose were intermediate relative to the low and high affinity sensors, with $\sim 50\%$ of the external concentration.

Taken together, reliable quantification of FRET changes was possible even in the low expressing wild-type transformants.

Conclusions

Here, a detailed protocol is provided describing the application of FRET-based nanosensors for DIFI measurements in roots of wild-type A. thaliana ecotype Col-0. FRET imaging of seedlings at an early plant age circumvents silencing and allows the use of FLIPglu nanosensors. The protocol described here will potentially be useful for analysis of FRET sensors in plant species for which silencing mutants are not available, or if analysis of silencing mutants is considered problematic due to potential effects of the silencing pathway on the process under analysis. Since gene silencing is probably caused by the cauliflower mosaic virus 35S promoter, another way of circumventing silencing might be the use of cell- or tissue-specific promoters.

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References


Takanaga H, Chaudhuri B, Frommer WB. 2008. GLUT1 and GLUT9 as major contributors to glucose influx in HepG2 cells identified by a high sensitivity intramolecular FRET glucose sensor. Biochimica et Biophysica Acta 1778, 1091–1099.


