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FRET-based glucose imaging identifies glucose signalling in response to biotic and abiotic stresses in rice roots

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ABSTRACT

Glucose is the primary energy provider and the most important sugar-signalling molecule, regulating metabolites and modulating gene expression from unicellular yeast to multicellular plants and animals. Therefore, monitoring intracellular glucose levels temporally and spatially in living cells is an essential step for decoding the glucose signalling in response to biotic and abiotic stresses. In this study, the genetically encoded FRET (Förster resonance energy transfer) nanosensors, FLIPglu–2 μΔ13 and FLIPglu–600 μΔ13, were used to measure cytosolic glucose dynamics in rice plants. First, we found that the FRET signal decreased in response to external glucose in a concentration-dependent manner. The glucose concentration at which the cytosolic level corresponded to the $K_{0.5}$ value for FLIPglu–2 μΔ13 was approximately 10.05 μM, and that for FLIPglu–600 μΔ13 was 0.9 mM, respectively. The substrate selectivity of nanosensors for glucose and its analogues is D-Glucose > 2-deoxyglucose > 3-O-methylglucose > L-Glucose. We further showed that the biotic elicitors (flg22 and chitin) and the abiotic elicitors (osmotic stress, salinity and extreme temperature) induce the intracellular glucose increases in the detached root segments of transgenic rice containing FLIPglu–2 μΔ13 in a stimulus-specific manner, but not in FLIPglu–600 μΔ13 transgenic lines. These results demonstrated that FRET nanosensors can be used to detect increases in intracellular glucose within the physiological range of 0.2–20 μM in response to various stimuli in transgenic rice root cells, which indicated that intracellular glucose may act as a potential secondary messenger to connect extracellular stimuli with cellular physiological responses in plants.

1. Introduction

Glucose is the predominant source of carbon energy and the most important sugar-signalling molecule, regulating metabolites and modulating gene expression from unicellular yeast to multicellular plants and animals. Extracellular glucose levels in yeast (*Saccharomyces cerevisiae*) are sensed by the Snf3 and Rgt2 glucose sensors, which are localised on the cell surface. Then, the signal is transduced into the nucleus, activating Rgt1 transcription factor to regulate the transcription expression of glucose transporters via a signalling transduction pathway shared by many kinds of tumour cells (Johnston and Kim, 2005; Gatenby and Gawlinski, 2003). In plants, glucose contributes to a wide range of physiological processes, including germination, seedling development, photosynthesis, carbon and nitrogen metabolism, flowering and seed filling, senescence, and responses to stress and pathogen infection (Chen et al., 2015a; Rolland et al., 2006). In *Arabidopsis thaliana*, it was shown that the regulator of G-protein signalling protein 1 (AtRGS1) as a putative extracellular glucose sensor forms a complex with the heterotrimeric G protein to regulate steady-state levels of transcripts from a small set of sugar-regulated genes in a G-protein-coupled signalling network (Grigston et al., 2008). Despite the essential roles of extracellular glucose as a primary messenger were sensed and utilised in relation to cellular metabolic status and biotic or abiotic stress responses (Lastdrager et al., 2014), the molecular mechanisms of glucose signalling pathways remain elusive in plants and mammals.

The spatial and temporal distribution of glucose in plant cells is a foundation for understanding glucose signalling transduction. Using a non-aqueous fractionation method, about 77%, 14.3%, and 8.7% of glucose was identified in vacuoles, cytosol, and the plastid of potato tubers, respectively (Farré et al., 2001). In the leaves of tobacco, up to

Abbreviations: eCFP, enhanced cyan fluorescent protein; eYFP, enhanced yellow fluorescent protein; FRET, Förster resonance energy transfer; 2-dG, 2-deoxyglucose; 3-OMG, 3-*O*-methylglucose; chitin, chitooctaose octahydrochloride; flg22, flagellin 22

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98% of hexose was detected in the vacuole (Heineke et al., 1994). It was further shown that cytosolic glucose concentrations in *Arabidopsis* root cells are < 90 nM in the absence of photosynthesis or an external supply (Deuschle et al., 2006). These results suggested that the vacuole provides a glucose pool involved in glucose metabolism and signalling transduction. Servaites and Geiger (2002) have demonstrated that glucose influx and efflux occur via the inner envelope of plastids, which suggests that some glucose transporters are involved in these processes. SWEET2 as an *Arabidopsis* sugar transporter was identified to be localised to the tonoplast, where it took the role of sequestering glucose in vacuoles and thereby limiting carbon efflux in the cortex and epidermis of roots (Chen et al., 2015a). AtVGT1 (*Arabidopsis thaliana* glucose transporter 1) was localised in the vacuolar membrane, where it was required for the osmotic adjustment and turgor increase during seed germination and flowering (Aluri and Büttner, 2007). SWEET8/RPG1 as a glucose transporter was indicated to be localised to the plasma member, where it was required for exine pattern formation of microspores in *Arabidopsis* (Guan et al., 2008). To date, three classes of eukaryotic sugar transporters, glucose transporters, sodium–glucose symporters, and SWEETs, have been characterised in multicellular organisms (Chen et al., 2015b). Thus, various sugar transporters localised in different membranes contribute to the distribution of glucose in cellular compartments.

Cytosolic glucose can be sensed by hexose kinase (HXK), which is a moonlighting protein involved in glucose metabolism and signalling to regulate growth and development in yeast, mammals, and plants (Dentin et al., 2004; Granot et al., 2013; Moreno et al., 2005). It was proved that *Arabidopsis* HXK1 as an intracellular glucose sensor evokes a response to the changing environment by uncoupling glucose sensing from its phosphorylation with HXK1 mutants lacking catalytic activity, thereby connects nutrient, light and hormone signalling networks for controlling growth and development (Moore et al., 2003). Cho et al. (2006) further demonstrated that HXK1 directly modulates glucose-regulated gene transcription independent of glucose metabolism in *Arabidopsis*, which mainly depends on a glucose signalling complex core with two unconventional, nucleus-specific HXK1 partners, vacuolar H^+-ATPase B1 and the 19S regulatory particle of the proteasome subunit. In addition, OsHXK5, OsHXK6, and OsHXK7 in rice, and NtHXK1 in tobacco, as glucose sensors can complement the *Arabidopsis glucose insensitive2-1* (*gin2-1*) phenotype (Cho et al., 2009; Kim et al., 2013; Kim et al., 2016). Thus, the intracellular glucose and its sensors represent core components involved in decoding the glucose signalling transduction pathway. However, measuring cytosolic glucose levels in living cells remains problematic due to their highly dynamic fluctuation. Using nuclear magnetic resonance spectroscopy and/or gas chromatography mass spectrometry, the distribution of ^{13}C-labelled glucose isotopologs can be used to analyse the labelling patterns by various models for deducing metabolic fluxes (Edwards et al., 1998; Ettenhuber et al., 2005). However, the spatial resolution and subcellular dynamics of the glucose in living cells are limited.

The development of genetically encoded FRET (Förster resonance energy transfer) nanosensors has significantly aided the study of cytosolic glucose dynamics at high spatial and temporal resolution in mammals, yeast, and *Arabidopsis* (Bermejo et al., 2010; Deuschle et al., 2006; Fehr et al., 2003). A set of FRET glucose sensors with different glucose affinities ($K_{0.5}$), covering the low nano- to mid-millimolar range, were produced by the fusion of glucose/galactose-binding protein MglB with a pair of green fluorescent protein variants, an enhanced cyan fluorescent protein (eCFP) and an enhanced yellow fluorescent protein (eYFP) (Deuschle et al., 2005). In *Arabidopsis*, the sensors FLIPglu-170nΔ13, −2 μΔ13, −600 μΔ13, and −3.2mΔ13 were used successfully to monitor steady-state levels of glucose and their changes in response to an external glucose supply in the leaf epidermis or roots (Chaudhuri et al., 2008, 2011; Deuschle et al., 2006). To clarify the physiological roles of glucose as a signalling molecule in rice, it is necessary to use FRET nanosensors to monitor the dynamic level of cytosolic glucose in living cells of rice plants. In this study, we built constructs with FLIPglu–2 μΔ13 and FLIPglu–600 μΔ13 expression driven by UBIQUITIN promoters to express each of these two glucose nanosensors to detect cytosolic glucose changes in detached rice root segments exposed to various biotic and abiotic stresses. Our results indicated that the dynamics of cytosolic glucose detected with FRET nanosensors are within the physiological range in response to different stimuli, suggesting that cytosolic glucose may act as a signalling molecule or a secondary messenger to connect extracellular stimuli with cellular physiological responses in rice plants.

2. Materials and methods

2.1. Rice plant growth

Rice plants (*Oryza sativa* L. spp. *japonica* cv. Zhonghua11) were planted in a growth chamber and in fields (from May to October, annually) at Beijing Normal University (Beijing, China). Transgenic rice plants were regenerated from the transformed calli on selection media containing 50 mg/L hygromycin and 250 mg/L cefotaxime after the calli had been incubated with *Agrobacterium tumefaciens GV3101* containing FLIPglu–2 μΔ13 or FLIPglu–600 μΔ13. To produce homozygous transgenic rice lines (T_3), transgenic plants were selected by 3:1 separation in the growth chamber and grown in the field for several generations. The transgenic lines were grown in the chamber under a 14-h light (24 °C)/10-h dark (20 °C) photoperiod and relative humidity of 85%. After culturing for approximately 10 days in Yoshida's culture medium (Yoshida et al., 1976) that was replaced each day, the seedlings were ready for glucose imaging.

2.2. Chemical reagents

All chemicals except those mentioned elsewhere were purchased from Sigma-Aldrich (St. Louis MO, USA). Flg22 was dissolved in dimethylsulfoxide to make a 1 mM stock solution. Chitooctaose (Chitin) purchased from Qingdao BZ Oligo Biotech Co. Ltd (Qingdao, China) was dissolved in water to make a 1 mM stock solution and kept at −20 °C. Before use, the flg22 and Chitin stock solution were diluted in Yoshida's culture solution to 100 nM. Sorbitol and NaCl were dissolved in Yoshida's culture solution to 250 or 300 mM, respectively. D-glucose (D-Glu), 2-deoxyglucose (2-dG), 3-O-methylglucose (3-OMG), and L-Glucose (L-Glu) were respectively dissolved in water to make 1 M stock solutions, which were diluted in Yoshida's culture solution to the appropriate concentrations prior to use.

2.3. Vector constructs for expression in rice

The plasmids, *pFLIPglu–2 μΔ13* and *pFLIPglu–600 μΔ13,* were purchased from Addgene (http://www.addgene.org/, plasmid ID 12990 and 12991). The DNA fragments of *FLIPglu–2 μΔ13* and *FLIPglu–600 μΔ13* were amplified and ligated into pTCK303 (Wang et al., 2004) via the restriction enzyme sites Kpn I/Spe I flanking the cassette. The plasmids were introduced into *Agrobacterium tumefaciens GV3101* and prepared for rice transformation. The primer sequence of *FLIPglu* used for the PCR amplification is provided in Table S1, and the construct maps for *FLIPglu–2 μΔ13* and *FLIPglu–600 μΔ13* are presented in Supplementary Fig. S1. The vectors were verified by DNA sequencing.

2.4. Expression analyses of FLIP glucose nanosensors in transgenic rice

Total RNA was prepared from one-week-old transgenic seedlings using TRIzol® reagent (Life Technologies, USA) and purified using a PureLink® RNA Mini Kit (Invitrogen, USA) combined with a PureLink® DNase Kit (Invitrogen), in accordance with the manufacturers' protocols. RNA concentration and quality were measured using a NanoVue