Conversion of a Putative Agrobacterium Sugar-binding Protein into a FRET Sensor with High Selectivity for Sucrose

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Glucose is the main sugar transport form in animals, whereas plants use sucrose to supply non-photosynthetic organs with carbon skeletons and energy. Many aspects of sucrose transport, metabolism, and signaling are not well understood, including the route of sucrose efflux from leaf mesophyll cells and transport across vacuolar membranes. Tools that can detect sucrose with high spatial and temporal resolution in intact organs may help elucidate the players involved. Here, FRET sensors were generated by fusing putative sucrose-binding proteins to green fluorescent protein variants. Plant-associated bacteria such as Rhizobium and Agrobacterium can use sucrose as a nutrient source; sugar-binding proteins were, thus, used as scaffolds for developing sucrose nanosensors. Among a set of putative sucrose-binding protein genes cloned in between eCFP and eYFP and tested for sugar-dependent FRET changes, an Agrobacterium sugar-binding protein bound sucrose with 4 μM affinity. This FLIPsucc-4μ protein also recognized other sugars including maltose, trehalose, and turanose and, with lower efficiency, glucose and palatinose. Homology modeling enabled the prediction of binding pocket mutations to modulate the relative affinity of FLIPsucc-4μ for sucrose, maltose, and glucose. Mutant nanosensors showed up to 50- and 11-fold increases in specificity for sucrose over maltose and glucose, respectively, and the sucrose binding affinity was simultaneously decreased to allow detection in the physiological range. In addition, the signal-to-noise ratio of the sucrose nanosensor was improved by linker engineering. This novel reagent complements FLIPs for glucose, maltose, ribose, glutamate, and phosphate and will be used for analysis of sucrose-derived carbon flux in bacterial, fungal, plant, and animal cells.

Sucrose is the major transported form of carbohydrates in plants. Also, many microorganisms including the yeast Saccharomyces cerevisiae use plant-derived sucrose as a carbon and energy source. Yeast uses both secreted and cytosolic invertases to hydrolyze sucrose and take up the resulting hexoses. In the mammalian intestine sucrose is cleaved extracellularly and taken up in the form of hexoses as well. The transport mechanism of sucrose in plants has been studied, and H+ -sucrose co-transporters have been cloned and characterized (1). Even though much has been discovered about phloem import of sucrose, essentially nothing is known about the export from photosynthetic mesophyll cells. Although it is conceivable that the H+-coupled transporters can work in reverse for exporting sucrose from the phloem in “sink” tissues, direct evidence of this as the major export route is lacking (2, 3). Perhaps more importantly, the microscopic distribution of sucrose within cellular compartments is largely unknown. Many components of complex sugar signaling networks have been discovered in yeast, but little progress has been made in identifying the corresponding players in plants. Proteomics has identified candidates for sugar transport across tonoplast membranes, but direct proof for a function in sugar transport across the tonoplast membrane is still lacking (4). Evidence has been presented that sucrose is transported across the plastid membranes, but the transporters are not known (5). Thus multiple transporters still await identification and characterization, and the regulation of sucrose transport both across the plasma membrane and among compartments is not well understood. Methods and reagents for real-time tracking of sucrose and its metabolites in living tissue with subcellular resolution would facilitate analysis of these fundamental questions; unfortunately, no currently available technology addresses these issues in a satisfactory manner. Non-aqueous fractionation is static and invasive, has no cellular resolution, and is sensitive to artifacts (6). Spectroscopic methods such as nuclear magnetic resonance imaging and positron emission tomography provide dynamic data but have poor spatial resolution and sensitivity.

The development of genetically encoded molecular FLIPs (fluorescent indicator proteins; also termed nanosensors as distinct from endogenous “sensor” proteins, which regulate response to environmental factors), which transduce the interaction of a target molecule with a recognition element into a macroscopic observable via allosteric regulation of one or more reporter elements, offers potential solutions to all of the above issues. Recognition elements are taken from the set of all binding proteins, including receptors and enzymes and protein or peptide ligands and substrates of other proteins, as in the use of a specific target sequence in the construction of a sensor for protease activity (7). Sterically separated donor-acceptor FRET pairs of fluorescent proteins (green fluorescent protein spectral variants or otherwise) can be used as spectroscopic reporter elements (8).

We have recently constructed a family of genetically encoded FRET sensors (all without conformational actuator) and demonstrated the generalizability of the method to a variety of ana-
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lytes. Members of the bacterial periplasmic-binding protein (PBPs)
superfamily recognize hundreds of ligands with high affinity (atto- to low micromolar) and specificity (9). PBPs have been shown by a variety of experimental techniques to undergo a significant conformational change upon ligand binding; direct linear fusion of an individual sugar binding PBP with a pair of green fluorescent protein variants has produced FLIPs for maltose, glucose, and ribose (8, 10, 11). The expression of the sensors has been shown to be sufficient for good signal-to-noise in yeast, animal, and plant cells (8, 12, 13). Conversely, expression has not been high enough to grossly perturb metabolism, as deduced from normal growth characteristics and indistinguishable phenotype. The nanosensors were used to measure sugar uptake and homeostasis in living animal cells, to determine subcellular analyte levels with nuclear-targeted versions (14), and to elucidate the presence of a high capacity bi-directional glucose transport system across the lumen of the endoplasmic reticulum (12). Moreover, using transgene-silencing mutants the glucose nanosensors were successfully expressed in plants and used to determine steady-state glucose levels in leaf epidermis and intact roots (13). In roots, sensors with affinities of 170 nm, 2 μm, and 600 μm responded to perfusion of the organs with glucose. Interestingly, steady-state levels in roots dropped below 100 nm; thus at least 100,000-fold lower than expected based on other studies. The availability of FRET-based sucrose nanosensors would complement this set of tools by facilitating the determination of steady-state sucrose levels and the kinetics of sucrose uptake, metabolism, and compartmentation.

Plant-associated bacteria can take up α-glucosides such as sucrose and α-galactosides such as raffinose via ABC transporters with the help of periplasmic-binding proteins (15, 16). These sugars may serve as nutrient sources or as osmo-protectors for bacteria growing in the rhizosphere of their host plants. Disaccharide uptake seems to be critical for bacterial colonization of plant roots (17). The expression of the putative α-galactoside-binding protein AgpA from Rhizobium meliloti is induced by α-galactosides (16). The aglEFGK operon from Sinorhizobium meliloti encodes an α-glucosidase and a PBP-dependent transport system for α-glucosides, particularly sucrose, trehalose, and maltose (18), which is inducible by sucrose and trehalose (17, 18). The ThuEFGK operon of S. meliloti encodes a similar set of genes, including a binding protein SmThuE and a transporter of sucrose, trehalose, and maltose (17). A mutant defective in thuE was impaired in growth on trehalose and maltose, whereas growth on sucrose and glucose appeared unaffected. Cosmid-borne Agl genes mediate uptake of radio-labeled sucrose into Ralstonia eutropha cells (18). Although no direct evidence has been provided yet, it is likely that the binding proteins AgpA, AglE, and ThuE recognize sucrose. Because plants contain high levels of sucrose but lower levels of maltose, trehalose, or other glucosides, the Thu and Agl systems may have overlapping specificities for disaccharides, and it is suspected that the Thu system recognizes trehalose with highest affinity, whereas the Agl system may be more important in sucrose transport (17, 18). A redundant set of sugar-transport operons may allow for optimal import of carbohydrates from the environment, with the relative affinities tuned to sugar abundance.

Therefore, SmAglE, SmThuE, and its homologues were tested by linear fusion to eCFP and eYFP for their suitability as scaffolds for the development of a FLIP nanosensor for sucrose. This system provides a reliable assay for specificity and was used to identify a chimera able to bind sucrose. As suggested by the potential functional overlap of the Thu and Agl operons, the nanosensor recognizes a spectrum of α-glucosides. Rational design on the basis of a homology model was used to improve the selectivity of the nanosensor for sucrose.

EXPERIMENTAL PROCEDURES

FLIPSuc Constructs and Plasmids—A PCR product from Agrobacterium tumefaciens (strain C58C1) genomic DNA encoding mature AtThuE without a stop codon was cloned into the KpnI site between eCFP and eYFP genes replacing EcngBlu in the FLIPglu-600μ cassette (10). The chimeric gene was inserted into pSET (Invitrogen) and transferred to Escherichia coli BL21(DE3)Gold (Stratagene). The AtThuE sequence was confirmed by DNA sequencing and was found to carry a N192D substitution relative to the published sequence. Because N192D is outside the binding pocket in the modeled structure and since AtThuE carrying the mutation is functional as a sugar nanosensor (see Tab. 1), all further experiments were carried out with the FLIPSuc carrying the mutation N192D, dubbed FLIPSuc-4μ. Mutants with F113A, D115A, D115E, D115N, W244A, Y246A, and W283A substitutions were generated using Kunkel mutagenesis (19) in the FLIPSuc-4μ background. Constructs with shortened linkers between the green fluorescent protein variants and ThuE were created from FLIPSuc-90μ. FLIPSuc-90μΔ1 was created by sub-cloning AtThuE into FLIPglu-600μΔ5 (20) with KpnI, which created a construct with 13 amino acids deleted from the linkers. FLIPSuc-90μΔ2 was created by shortening the linkers to the same length as in FLIPglu-600μΔ13, yielding a nanosensor with linkers 25 amino acids shorter (20). FLIPSuc proteins were extracted from BL21(DE3)Gold and purified as described (8).

In Vitro Characterization of FLIPSuc—Ligand titration curves and ligand specificity analysis were performed on a Safire (Tecan) fluorimeter. eCFP was excited at 433 nm, with emission maxima of 485 and 528 nm for eCFP and eYFP, respectively (bandwidth 12 nm). FRET was determined as eYFP/eCFP emission intensity ratio. Using the change in ratio upon ligand binding, the Kd of each FLIP was determined by fitting ligand titration curves to a single-site binding isotherm: S = (r − rapo)/(r sat − rapo) = [L]/(Kd + [L]), where S is saturation, [L] is ligand concentration, r is ratio, r apo is ratio in the absence of ligand, and r sat is ratio at saturation with ligand. Analyses were carried out with at least two independent protein preparations; for each protein at least two titrations were performed.

Homology Modeling—In the absence of structural information for AtThuE, a homology model was created based on the sequence similarity with the maltose/trehalose-binding protein of Thermococcus litoralis (TlMalE; solved in complex with tre-

2 The abbreviations used are: PBP, periplasmic-binding protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; MOPS, 4-morpholinepropanesulfonic acid.
halose; PDB code 1EU8; sequence alignment, Fig. 1; binding pocket model, Fig. 4). Protein side chains in the binding pocket were modeled using a backbone-independent rotamer library (23) and a semi-empirical force field (24); modeling was performed in the presence of the trehalose conformer from the 1EU8 structure. Structural models were made for maltose and sucrose by taking coordinates from the *E. coli* maltose-binding protein (PDB code 1ANF) and a sucrose-specific porin (PDB code 1A0T), respectively, and generating a small ensemble of ligand conformers by uniform sampling around the two rotatable disaccharide bonds. These ligand ensembles together with a model of glucose taken from the non-anomeric glucosyl moiety of the trehalose ligand of the 1EU8 structure were superimposed onto trehalose using the non-anomeric glucosyl as a template. For each ligand, poses sterically clashing with the modeled binding pocket were removed, leaving a single best conformer for each sugar (Fig. 4). All modeling was performed with in-house software (to be published).

RESULTS

Determining the Ligand Binding Specificity of a Putative Sugar-binding Protein—The putative *S. melliloti* disaccharide-binding protein SmThuE (GenBank™ AAD51827) was flanked with two green fluorescent protein variants by attaching a cyan (eCFP) and a yellow fluorescent protein (eYFP) to the N and C termini, respectively. The isolated fusion protein exhibited no discernible FRET, suggesting that protein stability may have been compromised. We have previously observed that within a binding protein family, only a subset may be facilely converted to working FRET sensors by simple linear fusion of chromophore variants; putative nanosensors may fail to show detectable FRET or show FRET but no ligand-dependent FRET change. SmAgIIE was fused in a similar manner but provided a FLIP with an extremely low starting ratio of ~0.7 and a sucrose-induced ratio change of only 0.05 (data not shown). Data base searches identified an *A. tumefaciens* protein closely related to SmThuE (GenBank™ NP_533835) (Fig. 1). AtThuE and SmThuE are 81% identical over the predicted open reading frames. SignalP 3.0 was used to predict the secretion signal sequences. AtThuE is also homologous to the malE maltose-binding proteins from *E. coli* and *T. litoralis* (*E. coli*, 21% identical; *T. litoralis*, 34% identical; both structures are known; Fig. 1). AtThuE was converted into a linearly fused FRET sensor by expression as a fusion between N-terminal eCFP and C-terminal eYFP fluorophores as before (Fig. 2A). The resulting chimera (designated FLIPSuc-4) functioned as a viable sucrose nanosensor, showing a sucrose-dependent decrease in eYFP/eCFP FRET ratio of 0.2 (Fig. 2, B and C; Table 1: apo-, 1.9; sucrose-saturated, 1.7), a Hill coefficient of 1.04, and a sucrose binding affinity of 4 μM.

Due to the sequence homology with the *E. coli* maltose-binding protein, one might naively expect that the AtThuE nanosensor would behave similarly to the previous maltose nanosensor, which showed a maltose-dependent increase in FRET efficiency, consistent with the model of the fluorophore closer proximity in the closed, relative to the open conformation (8). Point mutations and single amino acid deletions in the

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**FIGURE 1. Alignment of the sugar-binding protein sequences.** The amino acid sequences of the mature AtThuE (NP_533835), SmThuE (AAD51827), TlMalE (AAC38136), and EcmAle (AABS9056) were aligned using ClustalW in DNASTar. Mature proteins had been predicted by SignalP. Conserved positions are boxed. Residues involved in trehalose binding in TlMalE are marked; *+, interactions primarily with the non-reducing glucose; *, interactions primarily with the reducing glucose.

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3 H. Gu, S. Lalonde, S. Okumoto, L. L. Looger, A. M. Scharff-Poulsen, A. R. Grossman, J. Kossmann, I. Jakobsen, and W. B. Frommer, submitted for publication.
binding protein-fluorophore linker sequences can alter both the sign and magnitude of the signal change (20); it thus appears that more subtle effects than the macroscopic change in fluorophore separation contribute to the observed signal change.

**Ligand Binding Specificity of FLIPsuc**—The determination of ligand concentrations in complex mixtures, e.g. the cytoplasm of living cells, requires nanosensors with high specificity for their target ligand. The AtThuE-based FLIPsuc nanosensor was screened on the basis of its sucrose binding affinity; subsequently, a panel of related sugars was used to determine the specificity of the nanosensor. When incubated with 20 μM sugar, the FLIPsuc-4 μ nanosensor showed a similar signal change for sucrose, maltose, trehalose, and turanose (Fig. 3). The nanosensor responded to glucose and palatinose but with a lower ratio change, indicating a lower binding affinity or altered ligand-bound closed conformation. Galactose, lactose, melibiose, raffinose, and cellobiose did not give rise to ligand-dependent FRET changes even at concentrations of 50 times the sucrose affinity. Of the potentially interfering ligands, maltose and glucose were estimated to be present in the greatest concentration inside plant cells and, thus, were submitted to more careful analysis; trehalose levels in leaves are below the detection level under normal conditions (22). Trehalose was, therefore, not tested further. Full ligand binding titrations were performed for sucrose, maltose, and glucose (Fig. 2C, Table 1); maltose bound an order of magnitude more tightly than sucrose (230 nM), whereas glucose bound an order of magnitude more weakly (27 μM). These results are consistent with the predicted role of ThuE in maltose uptake and with the lower affinity of a disaccharide-binding protein for a monosaccharide.

**FLIPsuc Mutants with Altered Ligand Binding Affinity and Specificity**—Rational protein engineering was employed to increase the specificity of the FLIPsuc nanosensor for sucrose over the competing sugars maltose and glucose by altering the side chains directly interacting with the carbohydrate ligands. A homology model of AtThuE was generated based on sequence similarity to a protein of known structure. Interacting side chains were identified by visual inspection of the model, and mutations were proposed to alter binding to the three sugars (cf. “Experimental Procedures” and Fig. 4).

![Figure 2](image)

**Figure 2.** Characterization of FLIPsuc-4 μ. A, FLIPsuc construct with AtThuE flanked by eCFP and eYFP. B, emission spectra of FLIPsuc-4 μ in the presence and absence of 200 μM sucrose (excitation 433 nm). C, binding isotherms for FLIPsuc-4 μ for maltose, sucrose, and glucose. Ligand-dependent ratio changes have been transformed into sensor saturation. K_d values were determined as 3.7 μM for sucrose, 0.23 μM for maltose, and 27 μM for glucose. All titration curves were done in 20 mM MOPS, pH 7.0. Saturation curves were normalized to the ratio in the apo state.

**Table 1**

| Nanosensor       | Mutation | Sucrose K_d (μM) | Δ Ratio | Maltose K_d (μM) | -Fold | Δ Ratio | Glucose K_d (μM) | Δ Ratio |
|------------------|----------|------------------|---------|------------------|-------|---------|-----------------|---------|
| FLIPsuc-4 μ      | Wild type| 3.7              | 0.18    | 0.23             | 0.5   | 0.21    | 27              | 0.18    |
| FLIPsuc-10 μ     | F113A    | 10               | 0.12    | 0.12             | 0.5   | 0.12    | 106             | 0.12    |
| FLIPsuc-90 μ     | W283A    | 88               | 0.28    | 265              | 1,150 | 0.25    | 7,210           | 0.21    |
| FLIPsuc-1 m      | D115A    | 1,350            | 0.68    | 51               | 220   | 0.37    | 16,200*         | 0.50    |
| FLIPsuc-3 m      | D115E    | 2,870            | 0.36    | 1,550            | 6,600 | 0.39    | 9,110           | 0.44    |
| FLIPsuc-15 m     | Y246A    | 14,600*          | 0.14    | 56               | 240   | 0.14    | 554             | 0.07    |
| FLIPsuc-46 m     | W244A    | 46,200*          | 0.4     | 26,500*          | 115,200 | 0.42 | ND | ND |

*a Change in K_d for maltose compared to wild type (FLIPsuc-4 μ).

*b K_d estimated, could not reach saturation due to non-specific effects at high sugar concentrations; actual K_d may, thus, be different.
all eight proteins were purified from E. coli and tested for binding to sucrose, maltose, and glucose using the FRET assay (Fig. 5, Tables 1, 2). All seven mutants decreased affinity for all three sugars relative to the wild-type affinities, with the exception of a 2-fold increase in affinity of a single mutant (F113A) for maltose. A single mutant (D115N) abolished signaling for all three ligands, although whether binding affinity or the FRET signal transduction mechanism has been affected is unknown; this is the only protein position of the five in the “hinge” region of the binding pocket (23). It seems likely that the open-to-closed conformational change of this mutant nanosensor has been perturbed, as other sterically conservative mutations at this position do not abolish binding and sensing.

The aspartate side-chain Asp<sup>115</sup> is predicted to make good-geometry bidentate hydrogen bonds with both the HO2-hydroxyl of the non-anomeric glucosyl, and the HO3’-hydroxyl of the anemic glucosyl of maltose; it is modeled to make a similar hydrogen bond with the HO1’ hydroxyl of sucrose ( supplemental Fig. 1A). Mutation of this side chain to alanine has a modest 220-fold effect on maltose binding, suggesting solvent compensation for these hydrogen bonds; little effect on specificity is seen (less than 3-fold in all cases), consistent with the modeled equivalency of the hydrogen bonds. Mutation to glutamate has a more significant effect on maltose binding (6600-fold) and dramatically alters specificity. Specificity for glucose over sucrose is increased 2-fold and sucrose over maltose by 9-fold (Table 2). This is consistent with the modeled clash of the lengthened glutamate side chain on maltose binding (supplemental Fig. 1B), with less of an effect on sucrose, whose fructosyl group is modeled further away from this position, and even less of an effect on glucose, which lacks a second sugar ring. Importantly, this supports the model in which glucose, the glucosyl moiety of sucrose, and the non-anomeric glucosyl moiety of maltose occupy the same position in the binding pocket, making interactions with the eight side chains and one main chain segment that constitute the binding subsite for the non-reducing glucosyl moiety.

The mutation that affected ligand affinity most, after the non-signaling nanosensor carrying D115N, is W244A (10<sup>5</sup> decrease in maltose affinity), consistent with the prominent stacking interactions of this side chain with the non-anomeric glucosyl ring of all three ligands (supplemental Fig. 1C). This tryptophan side chain is also predicted to sterically interfere with the sucrose fructosyl, and as predicted, a 9-fold increase in sucrose specificity over maltose is observed in the alanine mutant. Glucose binding is below the detectable range for the W244A mutant.

The mutation Y246A leads to a modest 240-fold loss in maltose binding affinity and dramatically increases specificity for glucose over sucrose (11-fold) and for maltose over sucrose (16-fold). The cause for this specificity change is unknown; the tyrosine side chain is predicted to interact by weak stacking interactions equivalently well with the anomeric glucosyl of maltose and the fructosyl of sucrose (supplemental Fig. 1D). The large effect on glucose binding affinity hints at a substantial rearrangement of the binding pocket in this mutant.

The phenylalanine side chain Phe<sup>113</sup> is predicted to make close contacts with both the anomeric sugars of maltose and sucrose and is predicted to give rise to unsatisfied hydrogen bond acceptors and donors (O<sub>5</sub>’ and HO2’) in maltose, which it approaches more closely in the model (supplemental Fig. 1E) than the other sugars. Mutation of this side chain to alanine produces effects consistent with the model; maltose binding...
affinity is increased 2-fold, as solvent may now satisfy these hydrogen bonding groups, apparently more than compensating for the cavity created against the ligand. Sucrose and glucose, which are predicted not to have unsatisfied hydrogen-bonding groups, are modestly adversely affected, apparently by cavity formation.

The most dramatic effects on ligand binding specificity are seen in the W283A mutant. The tryptophan side chain of Trp283 is modeled to make the second-largest contact with maltose of any side chain (after Trp244) in a herring bone configuration relative to the fructosyl and glucosyl rings of sucrose and maltose, respectively (supplemental Fig. 1). Tryptophan 283 is predicted to interfere with the fructosyl moiety of sucrose via steric clashes with the atoms O3 and H11032 and O4 and H11032 and by preventing their hydrogen bond satisfaction (Fig. 4). Mutation of this side chain to alanine results in a nanosensor with a 103-fold decrease in maltose binding affinity, a 50-fold increase in specificity for sucrose over maltose, and an 11-fold for sucrose over glucose. This is consistent with the model of the sucrose sterically clashing with Trp283, truncation of which dramatically improves specificity. The tryptophan side chain must contribute positively to sucrose binding as well, as the overall effect on sucrose binding affinity is negative (20-fold decrease). This nanosensor, designated FLIPsuc-90μ, shows modest selectivity for sucrose over maltose (3-fold) and dramatic selectivity over glucose (80-fold). Given that sucrose levels in plant cells are predicted to be higher than for glucose (24, 25) and that cytosolic maltose levels are very low (<500 μM (26)) and trehalose levels are not detectable when trehalase is active (26), this nanosensor should provide adequate specificity to be useful as an in vivo sucrose nanosensor. The important question regarding the suitability of this sensor for in vivo measurements will be what the actual concentrations are in the cytosol. It has been suggested that sucrose levels are in the high millimolar range. Glucose levels have also been estimated in the millimolar range; however, the analysis of steady-state glucose levels using FLIPs has shown that in roots glucose levels can drop below 100 nM (13). Sensors with affinities for glucose of 170 nM, 2 μM, and 600 μM were suitable to measure changes in glucose concentrations in the cytosol in response to perfusion of the organs with glucose medium. These data suggest that also to FLIPsuc-90 μM will be in the correct range for in vivo measurements. Nevertheless it would be useful, especially to control for effects of other parameters such as pH or ionic conditions, to develop sensors with similar specificity but different affinities. Based on the structural model presented here, the affinity will be brought into the millimolar range with additional mutations to decrease affinity, hopefully without decreasing the specificity relative to maltose and glucose.

Optimization of the Signal-to-noise Ratio of the Sucrose Nanosensors—Binding site mutagenesis of the nanosensors had two beneficial effects; binding of the target ligand is improved relative to non-target ligands (specificity), and binding of the target ligand is moved into a more physiologically practical range (affinity). A third critical property of the FRET sensors

| Nanosensor | Mutation | Suc/Mal | Suc/Glu |
|------------|----------|---------|---------|
| FLIPsuc-1μ | F113A    | 0.2     | 1.5     |
| FLIPsuc-9μ | W283A    | 50      | 11      |
| FLIPsuc-1m | D115A    | 0.6     | 1.6     |
| FLIPsuc-3m | D115E    | 8.6     | 0.4     |
| FLIPsuc-15m| Y246A    | 0.06    | 0.005   |
| FLIPsuc-46m| W244A    | 9       |         |

FIGURE 5. Saturation curves of FLIPsuc mutants. Mutants were titrated with maltose, sucrose, and glucose. Each titration corresponds to two independent protein purifications, each used for two independent experiments. Concentrations higher than 100 mM were not used due to problems with nonspecific effects. Saturation curves were normalized to the ratio in the apo state.
may be simultaneously optimized by protein engineering; that of signal-to-noise. In a two-state system of ligand binding, the overall ligand-dependent signal change of a nanosensor will be the population-weighted difference in the ligand-free apo form and the ligand-bound closed form of the nanosensor (27). Alteration of the signal of the apo or the closed form or the relative population of these two states may either improve or lessen the overall nanosensor signal change. In the absence of an effect on the apo or closed state, mutations that decrease ligand binding affinity are expected to drive the conformational equilibrium toward the open state, thus magnifying the population difference between the ligand-free and ligand-saturated states, increasing overall signal change.

The seven purified nanosensors that showed ligand-dependent signal changes exhibit quite similar ligand-saturated FRET ratios for a given nanosensor with the three ligands (data not shown). The nanosensor with the best specificity, FLIPsuc-90μ, shows a signal-to-noise improved about 50% over the wild-type nanosensor but still insufficient for in vivo use, where signal-to-noise is almost always decreased (10, 12, 14).

It therefore seemed necessary to further improve the signal-to-noise of FLIPsuc-90μ while retaining the improved ligand binding specificity. Linker shortening has proven to be a reliable method for signal enhancement in a variety of nanosensor backgrounds (20). Two sets of amino acid deletions from the N- and C-terminal composite linkers connecting the binding protein with eCFP and eYFP (the composite linker includes poorly structured chromophore regions as well as any explicit linker sequence) were generated; they are a conservative deletion of 13 residues to create FLIPsuc-90μΔ1 and a more extensive deletion of 25 residues to create FLIPsuc-90μΔ2. FLIPsuc-90μΔ1 appears to have an unperturbed closed state, as measured by the FRET ratio proxy (Fig. 6); the ratio of the apo form was dramatically increased, improving the overall signal change ~4-fold over FLIPsuc-90μ, making it significantly more useful for in vivo measurements. The version carrying the complete linker deletion FLIPsuc-90μΔ2 showed an unchanged ratio change relative to FLIPsuc-90μ. The tentative placement of glucose, maltose, and sucrose in the binding pocket of the homology model provides a basis for generating further mutants with altered binding properties to expand the in vivo suitability.

**DISCUSSION**

To enable analysis of sucrose levels in living cells with subcellular resolution, a genetically encoded FRET sensor with specificity for sucrose was generated. Nanosensors for other sugars such as maltose and glucose have made use of the natural binding specificity of the PBPs, but specific sucrose-binding proteins have not yet been identified among the PBPs. It was hypothesized that microorganisms in the rhizosphere may have developed binding proteins for sucrose sensing and acquisition. It has been suggested that rhizobial proteins can bind a number of disaccharides including sucrose (17, 18). Fusion of candidate-binding proteins to eCFP and eYFP was used to identify a sucrose binder by measuring FRET changes in response to ligand addition. Several candidates from Rhizobium and Agrobacterium were tested, and a homologue of the rhizobial protein ThuE from A. tumefaciens was found to bind sucrose.

Because ThuE is known to be part of the maltose and trehalose transport and utilization pathway in S. meliloti (17), the new sucrose nanosensor was tested for ligand binding specificity. ThuE also bound maltose, trehalose, and turanose. Glucose and palatinose bound to the nanosensor but with lower affinity. Trehalose and turanose do not occur in high amounts in planta (22), and glucose and maltose were identified as the most likely interfering sugars for in vivo sucrose detection. A homology model of AtThuE in complex with each of sucrose, maltose, and glucose was created based on the structure of the T. litoralis maltose-binding protein in complex with trehalose (28), and residues predicted to interact with the ligand were mutated in an attempt to improve specificity. Most mutations led to a differential effect on the binding of the three sugars. Most mutations leading to increased selectivity for sucrose over glucose and maltose can be explained by the larger size and the altered position of the fructosyl moiety of sucrose relative to the anomeric glucosyl moiety of maltose. FLIPsuc-90μ discriminates sucrose 50-fold over glucose and 4-fold over maltose. This
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binding specificity in conjunction with the lower levels of glucose and maltose in the plant cytosol should enable accurate detection of sucrose in living cells such as yeast, intestinal cells, or in intact plants. Moreover, the maltose nanosensor FLIPmal and the glucose nanosensor FLIPglu (neither of which respond to sucrose) can serve as controls to evaluate maltose and glucose levels independently. Screening and selection methods have also been used to discover mutants of a maltose-binding protein with specificity for sucrose (21); it is likely that a combination of rational and empirical methods may prove to be a powerful technique for specificity engineering.

FLIPsuc exhibits a ratio change of ~0.2; of the binding-pocket mutations screened, only those in which residues in the hinge region have been mutantized show a higher ratio change. To enhance the sensitivity of the sucrose-selective nanosensor FLIPsuc-90μ, linker deletions were generated using the same rationale used for improving the glucose nanosensors. Deletion of the composite linkers, which consist of the artificially introduced flexible linker sequences as well as poorly structured hinge regions, have been deleted, shows an almost 4-fold increase in signal change. This nanosensor has a ratio change in a similar range as the improved glucose nanosensors (13, 20) and should be suitable for in vivo applications.

Glucose nanosensors have successfully been used in plants to study glucose levels in the cytosol of both roots and leaves (13). The results indicate that free glucose can accumulate in the cytosol when external supply is high but that steady-state glucose levels can be much lower than anticipated from nonaqueous fractionation studies, especially in roots where concentrations may drop below 100 nM, at least 100,000-fold below the levels described in leaves and tubers of Solanaceae. Depending on tissue and cell type, sucrose levels in plants were estimated in the range from low to high millimolar. The cytosolic sucrose concentration in developing potato tubers was estimated at 30 mM, whereas the levels of sucrose in the cytosol of maize leaves has been estimated as high as 850 mM (2, 25). As pointed out above, the results obtained with the glucose nanosensors (13) suggest that glucose concentrations are significantly below earlier estimates. Given that plant tissues contain high invertase activities in the apoplasm, the cytosol, and the vacuole, we hypothesize also that sucrose levels will drop in roots to at least to the low micromolar range. Therefore, we expect that FLIPsuc-90μ will be suitable for detection of steady-state levels in plant roots and leaves as well as in other organisms such as yeast or at the surface of intestinal cells to monitor sucrose flux. Because steady-state levels can be affected by any of the contributing fluxes (sugar import, and efflux at the plasma membrane, bio-synthesis, and metabolism as well as compartmentation), the sensors provide a means for detecting and differentiating environmental and regulatory processes that have an impact on any of the flux components. Moreover, flux is an indirect readout of the transcriptome and the proteome since it integrates over multiple levels of the “system,” it provides a new complementary tool to study fluxomics and systems biology.

Because all of the nanosensors developed so far have a limited in vivo detection range, it may nevertheless be useful to expand the spectrum of affinity mutants in the FLIPsuc-90μΔ1 background. The tentative placement of the sugars in the binding pocket will serve as a guideline for generating additional affinity mutants.

Many aspects of sucrose transport and regulation are poorly understood, e.g. export from mesophyll cells and vacuolar transport. The nanosensors may provide us with a tool to characterize these processes and to identify the underlying proteins and genes. Sucrose nanosensors could also be expressed in the same cell as glucose nanosensors with different FRET pairs, allowing simultaneous determination of sucrose and glucose flux. The FRET approach provides a simple means for identifying the ligand binding specificity of unknown binding proteins. Moreover, the availability of a FRET sensor for sucrose provides unique possibilities to characterize sucrose transport and the linked metabolic flux rates.

Acknowledgments—We thank Agnes Harms for constructing FLIPsuc-90μΔ2. We are very grateful to Sharon Long (Stanford) for providing genomic Rhizobium DNA and to T. V. Bhuvanveswari (Tromso, Norway) for providing plasmids and template DNA for SmThaE.

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