Journal of Biological Methods | **2014** | Vol. 1 | e5
DOI: 10.14440/jbm.2014.27

ARTICLE

Proof of concept for a novel functional screening system for plant sucrose effluxers

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Competing Interests: The authors have declared that no competing interests exist.

Received May 18, 2014; Revision received July 8, 2014; Accepted July 8, 2014; Published July 18, 2014

Abstract Membrane transporters play pivotal roles in facilitating sucrose transport in plants and their activities have been shown to impact plant growth rates and crop yield. In contrast to the well-defined mechanism of sucrose influx across plasma membranes, less is known about sucrose efflux mechanisms and the membrane proteins supporting this function. A major impediment blocking progress in this key area of plant science is the absence of a functional screening system for genes encoding sucrose effluxers. Here we report a novel yeast system for screening sucrose effluxers based on sucrose release from yeast cells genetically modified to synthesize, but not to metabolize, sucrose. Inhibiting sucrose metabolism was achieved using yeast strains, *SEY 6210* and *YSL4-6*, carrying mutations in genes encoding invertase and maltase, respectively. Genes encoding essential components of sucrose biosynthesis, sucrose phosphate synthase (*SPS*) and sucrose phosphate phosphatase (*SPP*), were used to transform the two yeast hosts to make strains *SuPy* (from *SEY6210*) and *Ysu* (from *YSL4-6).* Cultures of *SuPy15* cells were found to be capable of synthesizing sucrose when supplied with various compounds as the sole carbon source, including non-fermentable sugars and non-sugar substrates. A proof of concept of the screening system was demonstrated by transforming *SuPy15* with sucrose transporter genes known to encode plasma membrane proteins that mediate sucrose efflux. The robustness of the yeast *SuPy15* system as a novel platform to screen putative plant sucrose effluxers is discussed.

Keywords: functional screen, sucrose effluxer, sucrose transporter, yeast mutant, cDNA library

INTRODUCTION

Sucrose is the principal form in which plant biomass, produced by photosynthetic leaves (sources), is transported to non-photosynthetic organs (sinks) for growth and storage. Sucrose transporters contribute to this long-distance transport from source to sink by playing central roles in sucrose loading and unloading of the phloem transport system [1]. Sucrose transporters involved in the transmembrane steps influencing rates of phloem loading and unloading are of three types: proton/sucrose symporters located on plasma membrane responsible for sucrose entry into cells [1]; sugar/proton antiporters that load vacuoles across the tonoplast and sucrose/proton symporters that release sucrose back to the cytoplasm [2]; plasma membrane effluxers mediating sucrose exit from bundle sheath/phloem parenchyma cells in apoplasmic phloem loading of source leaf minor veins and possibly for facilitating sucrose unloading into sink organs [3]. Most progress has been made in understanding sucrose transporter (SUT) proteins responsible for sucrose influx across plasma membranes by proton/sucrose symport [1].

Genes encoding sucrose/H^+ symporters (SUTs) have been cloned from many plant species and functionally characterized [1]. The functional screen developed for cloning SUTs from plant cDNA libraries is the SuSy7 budding yeast (*Saccharomyces cerevisiae*) system [4]. SuSy7 is an invertase mutant transformed to express a plant sucrose synthase (SuSy) in the cytoplasm (**Fig. 1A**). Under these conditions, cDNAs of sucrose transporters complement SuSy7 otherwise unable to grow upon sucrose as the sole carbon source [4]. This robust system has led to identifying five major clades of plant SUTs, all of which function as sucrose/proton symporters [1]. The exceptions are a group of plasma membrane sucrose facilitators (SUFs) that support pH-independent and bi-directional sucrose transport [5] and members of the tonoplast monosaccharide transporter (TMT) family that have been functionally characterised as glucose and sucrose/proton antiporters supporting sugar efflux from vacuole to cytoplasm [6].

Compared with the large number of SUTs mediating sucrose influx, only a few transporters supporting sucrose efflux from cells across their plasma membranes have been identified to date [7]. A suite of SUFs (PsSUF1, PsSUF4 and PvSUF1), were cloned from legume seeds and functionally characterized to support sucrose efflux by their heterologous expression in SuSy7 [5]. Recently a new family of sucrose effluxers (SWEETs) was identified by FRET (Fluorescence resonance energy transfer) sugar sensors expressed in a human tissue culture cell line [3].

A major factor impeding progress in cloning sucrose effluxes is the lack of a high throughput and sensitive functional expression system capable of screening sucrose effluxers from cDNA libraries. In this context, *Saccharomyces cerevisiae* has proven to be an effective eukaryotic model for heterologous expression of membrane transporters. The ready availability of deletion mutants and a versatile set of selectable markers, combined with easy genetic manipulation have underpinned its wide use in high-throughput screening for drug targets [8]. More significantly, *S. cerevisiae* mutants have provided a convenient system for functional and kinetic studies of sucrose symporters [1].

In the current paper, we report the design of a novel functional cloning

system for sucrose effluxers based on detecting sucrose released to the medium from a transformed yeast strain capable of sucrose synthesis but not metabolism. We also provide proof of concept for the above system through its ability to support sucrose efflux when transformed with SUFs known to mediate sucrose efflux [5].

MATERIALS AND METHODS

Plasmid construction

The expression cassette, containing the PMA1 promoter, multiple cloning site (MCS) and ADH terminator was excised as a *Hind* III/*Sph* I fragment from the *pDR196*Δ, a *pDR196* vector with *EcoR* V, *Hind* III and *Sal* I sites deleted. The fragment was cloned into the *Hind* III/*Sph* I double-digested *pYIplac* 204Δ and *pYIplac 128*Δ*,* modified versions of yeast integrating vectors, *pYIplac 204* and *pYIplac 128* [9] due to the deletion of *Pst*I*, Sal*I*, XbaI, Bam*HI and *Sma*I sites. The resulting plasmids became *pYMA204*Δ and *pYMA128*Δ. The coding regions of the sucrose phosphate synthase gene from *Synechocystis, SynSPS (GenBank acc.* CP003265), and the sucrose phosphate phosphatase gene from tobacco, *NtSPP2* (AY729656) were PCR-amplified from *pDR197-NtSPP2* and *pBK-SynSPS* (both kindly supplied by Dr Furbank, CSIRO Plant Industry Canberra). The *NtSPP2* product was cleaved with *Sma*I and *Xho*I and cloned into the *Sma*I/*Xho* I double-digested *pYMA204*Δ to make *pYMA204*Δ*-NtSPP2*. The *SynSPS* product was cleaved with *Pst*I and *Xho*I and cloned into the *Pst*I/*Xho*I double-digested *pYMA128*Δ to make *pYMA128*Δ*-SynSPS.* The correct incorporation of identical full-length genes was verified by sequencing.

Yeast transformation and strain construction

S. cerevisiae strains *SEY 6210 (MATαleu2-3, 112 ura3-52 his-Δ200 trp1- Δ901 lys2-801 suc2- Δ9 GAL)* (Robinson et al 1988) and *YSL4-6 (MAT_ leu2-3,112 ura3-52 trp1-289 his3 Δ 1 Mal2-8c SUC2 hxt17 Δ hxt13 Δ::loxP hxt15 Δ::loxP hxt16 Δ::loxP hxt14 Δ::loxP hxt12 Δ::loxP hxt9 Δ::loxP hxt11 Δ::loxP hxt10 Δ::loxP hxt8 Δ:loxP hxt514 Δ ::loxP hxt2 Δ::loxP hxt367 Δ::loxP gal2 Δstl1 Δ::loxP agt1 Δ::loxP ydl247w Δ::loxP yjr160c Δ::loxP suc2 Δ::loxP)* were used as the parent strains to express sucrose synthesis genes. The strainYSL4-6 originated from the strain *EBY.VW4000* (CEN.PK2-1C *hxt17Δ hxt13Δ hxt15Δ hxt16Δ hxt14Δ hxt12Δ hxt9Δ hxt11Δ hxt10Δ hxt8Δ hxt514Δ hxt2Δ hxt367Δ gal2Δ slt1Δ agt1Δ ydl247wΔ yjr160cΔ*) [10] but with the invertase gene deleted (Lalonde, S unpublished). Transformation of the yeast strains was performed by the standard lithium acetate method [11]. The strain *SuPy* was constructed by co-transformation of strain *SEY 6210* with the *EcoR*V-digested *pYMA204*Δ*-NtSPP2* and *pYMA128*Δ*-SynSPS* plasmid DNA, whereas the strain *Ysu* was constructed by co-transformation of the strain *YSL4-6* with the two linearized DNA plasmids described above. Selection was performed on Leu and Trp drop-out SD agar (Sigma-Aldrich, Australia) supplemented with 2% glucose for *SuPy* and 2% maltose for *YSL4-6*. Five to ten individual colonies of *SuPy 10-19* and *Ysu 1-10* were selected from the initial transformation plates as biological replicates for each new strain. Colony PCR was performed using primers NtSPP624f (GGAGGAATTATTGCAATGGCATGCT-GC) and MCS2 (CCTCTGGCGAAGAAGTCCAAAGCTG) for the *NtSPP2* gene, and primers SynSPP1535f (ACTGGGAGAAGATTG-GATTCGGTGC) and MCS2 for the *SynSPP* gene. Selected colony PCR fragments were purified and sequenced to further confirm the identity of the clones. Colonies containing both verified *NtSPP2* and *SynSPS* genes were prepared as glycerol stocks for further assays.

Sucrose transporter genes, *PvSUT1*, *PsSUF1* and *PsSUF4*, cloned into yeast vector *pDR196* [5], were used to transform a selected clone of strain *SuPy, SuPy15* by the method described above. Colonies were selected on Ura-Leu-Trp drop-out SD agar (Sigma-Aldrich, Australia) supplemented with 2% glucose. Three to five individual colonies were selected and screened by colony PCR using gene specific primers for *PvSUT1*, *PsSUF1 and PsSUF4* [5]. Selected PCR fragments were further confirmed by sequencing.

Growth conditions for yeast strain *SuPy* and *Ysu*

Glycerol stocks (3-5 biological replicates per strain) were streaked onto SD drop-out plates supplemented with 2 % glucose or 2% maltose and grown at 30°C. Single colonies were used to inoculate 3 x 5-mL aliquots (technical replicates) of SD drop-out broth with either 2% glucose, 2% galactose, or 2% maltose in 50 mL Falcon tubes and cultured overnight at 30oC, 200 rpm. Overnight cultures were used to inoculate 10 mL SD dropout medium with appropriate sugars to an OD_{660} of 0.2. Cultures were monitored for growth by cell counts or sampled from mid- to late-log phase (5-7 x 10^7/mL) for the intracellular sucrose assay (see below). For yeast culture grown on non-sugar carbon sources, a single colony from a glucose plate was streaked onto an agar plate made up of SD drop-out medium supplemented with a specified carbon substrate (ethanol, glycerol or ethanol + galactose, ethanol + glycerol). Colonies from the plates were transferred to liquid SD drop-out broth with appropriate carbon substrates. The pre-culture was then diluted with appropriate fresh medium and monitored for growth or intracellular sucrose analysis. For the intracellular sucrose assay of cells cultured on agar plates, single colonies from SD drop-out plates, with appropriate substrates, were used to streak onto appropriate fresh medium plates and cultured until fully-grown colonies appeared. Cells were then scraped off each plate, washed twice with MilliQ H_2O and resuspended in MilliQ H_2O to a final cell concentration of 5-7 x 10^7 cells /mL for the sucrose assay.

Intracellular sucrose assay

A total of 5 mL of culture media was centrifuged (5000 × *g*, 4°C, 5 min) to pellet yeast cells that were then washed twice with ice-cold MilliQ H_2O and resuspended in 250 µL MilliQ H_2O. Cells were disrupted by heating at 85°C for 15 min. The disrupted cells were centrifuged at 13000 × *g* for 5 min and the supernatant collected. The released sucrose was detected by an enzyme-coupled assay [12].

Sucrose efflux assay

The *SuPy15* yeast cells were grown for 17 h in 500 µL SD drop-out medium containing 2% glucose. The medium contained 10 µCi D-[^{14}C]glucose/mL. Following centrifugation, the supernatant was collected, freeze-dried and re-dissolved in 50 µL ethanol. Aliquots of 10 µl were used for separation of extracellular sucrose by thin-layer chromatography with ethyl acetate: acetic acid : methanol : H_2O (60 : 15 : 15 : 10. v/v/v) as the mobile phase. Sucrose spots were identified by co-chromatography with standard chemicals, and scraped off plates for radio-assay to determine amounts of [^{14}C] according to [5].

RESULTS AND DISCUSSION

Developing a novel functional cloning system for sucrose effluxers

The yeast functional cloning system was designed to efflux (**Fig. 1B**), rather than influx, sucrose as performed by the SuSy7 strain (**Fig. 1A**).

This system requires a yeast strain able to produce and accumulate sucrose intracellularly. It is generally accepted that in yeast, sucrose

fermentation proceeds predominantly through an extracellular invertase that cleaves sucrose into glucose and fructose, followed by uptake and metabolism of these hexoses. Therefore, direct uptake of sucrose into yeast cells is much lower than that of its hydrolysis products. Furthermore, yeast invertase is encoded by one or several *SUC* genes, with each gene enabling synthesis of two isoforms of invertase, extracellular and intracellular [13]. While sucrose hydrolysis by *S. cerevisiae* predominantly occurs extracellularly, intracellular invertase is expressed constitutively at a low level [13]. Sucrose taken up into yeast cells also can be hydrolyzed by other intracellular glycosidases, such as maltase, an enzyme with the same affinity for sucrose and maltose [14]. Thus intracellular sucrose concentrations of wild type yeast are unlikely to be high enough to support sucrose release through an effluxer.

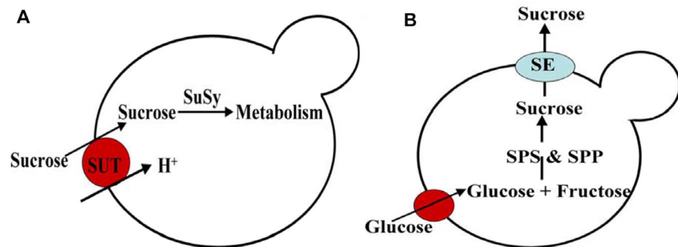

Figure 1. Comparison of the functional cloning systems for (A) sucrose influxers and (B) sucrose effluxers. A. Yeast line expressing a sucrose metabolizing enzyme (SuSy). Only those cells transformed with a plant sucrose/H+ symporter (SUT) able to survive on a sucrose medium as the sole carbon source. **B.** Yeast line expressing sucrose phosphate synthase (SPS) and sucrose phosphate phosphatase (SPP), enzymes that catalyse sucrose synthesis. Cells transformed with a plant sucrose effluxer (SE) will release sucrose to the medium. The concentration of extracellular sucrose can be measured in the medium.

To generate a yeast strain with the biochemical capability of synthesizing but not metabolizing sucrose, yeast strains *SEY 6210* and *YSL4-6* were selected as hosts (parent strains) for heterologous expression of key enzymes required for sucrose synthesis, sucrose phosphate synthase (SPS) and sucrose phosphate phosphatase (SPP). Both strains have multiple auxotrophic markers for gene manipulation. Strain *SEY6210* contains a complete deletion of the *SUC2* gene and no other unlinked invertase structural genes [15]. Strain *YSL4-6* originated from the strain *EBY.VW4000* with its entire hexose uptake system [10] and invertase genes deleted (Lalonde, S unpublished). A yeast strain lacking the capability for hexose uptake could be advantageous as hexose transporters can work in reverse under certain circumstances [16]. This would result in glucose leakage that could then interfere with detection of any effluxed sucrose. Transforming the two yeast strains with genes encoding sucrose phosphate synthase from *Synechocystis*, *SynSPS*, and sucrose phosphate phosphatase from tobacco, *NtSPP2*, conferred the capability for sucrose biosynthesis. The resulting strains of the two yeast hosts transformed with *SynSPS* and *NtSPP2* were named *SuPy* (from *SEY6210*) and *Ysu* (from *YSL4-6)*. After transformation, colony PCR demonstrated that the majority of colonies grown on Leu-Trp drop-out plates were positive for the presence of both *SynSPS* and *NtSPP2* (**Fig. 2**).

Strains *SuPy* and wild type *SEY6210* were grown on 2% glucose, while strain *Ysu* and the wild type *YSL4-6* were grown on 2% maltose, due to the deletion of its hexose transport system [10]. Growth rates of these strains were comparable based on identical cell numbers being reached during overnight culture (**Fig. 3**). Both wild type *SEY6210* and wild type *YSL4-6* contained negligible levels of intracellular sucrose supporting the claim that intracellular sucrose is low in *S. cerevisiae*. A similar outcome was obtained for all the tested clones of *Ysu*. In contrast, significant amounts of intracellular sucrose were accumulated in all tested clones of the *SuPy* strain (**Fig. 3**). Based on these findings, *SuPy15*, was selected for further characterization.

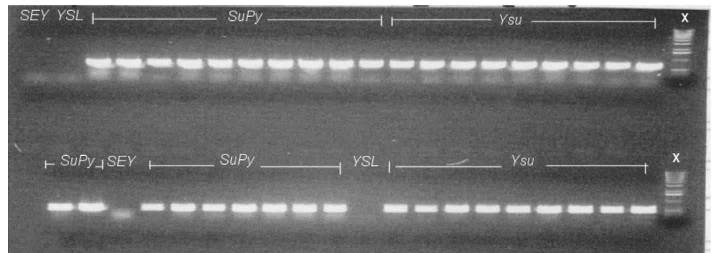

Figure 2. Screening of *SuPy* and *Ysu* strains by colony PCR. Top lanes, colony PCR screen for *SynSPS* gene, a 633 bp fragment; Bottom lanes, colony PCR screen for *NtSPP2* gene, a 607 bp fragment. SEY, *SEY6210* wild type; YSL, *YSL4-6* wild type; *SuPy*, *SuPy* colonies; *Ysu*, *Ysu* colonies; X, DNA marker X (Roche).

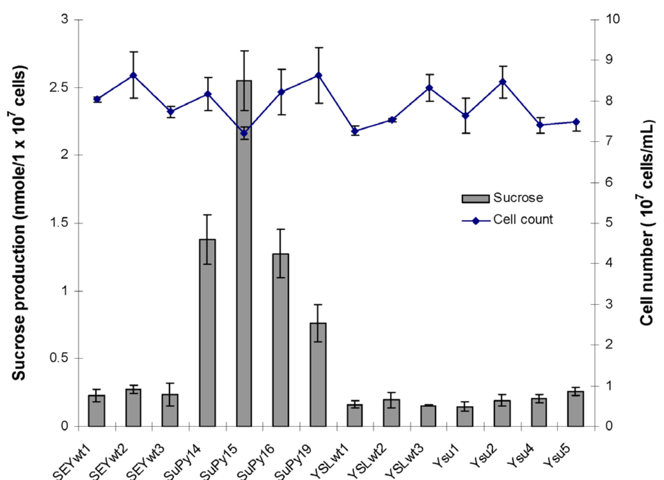

Figure 3. Sucrose production in yeast strains transformed with *SynSPS* and *NtSPP*. Yeast cultures were grown to a density of 7- 9 x10^7 cells/mL and harvested for intracellular sucrose assay. SEYwt1-3, wild type *SEY 6210* strain, clone 1-3; *SuPy* 14-16, 19, four clones of *SuPy* strain constructed by transformation of *SEY 6210* strain with sucrose biosynthetic genes; *YSLwt*1-3, wild type *YSL4-6* strain, clone 1-3; *Ysu1-5*, five clones of *Ysu* strain constructed by transformation of *YSL4-6* strain with the sucrose biosynthetic genes. All values represent the mean ± SE of three biological replicates.

Effect of carbon source on sucrose production of *SuPy15* strain

One of the advantages of growth on carbon sources other than glucose is to avoid any interference by glucose in detecting sucrose released to the medium (**Fig. 1B**). Apart from growth on glucose, the *SuPy15* strain grew well on galactose (**Table 1**). However, significantly slower growth was observed when non-sugar compounds, such as ethanol or glycerol, were used as the sole carbon source (**Table 1**). In contrast to culture with glucose or galactose, the growth condition here specifically included two media passages to minimize an otherwise extended lag phase preceding cell growth. The first passage was from a solid media containing glucose to a solid media containing a non-sugar carbon. The second passage was from a solid to liquid culture media containing the non-sugar carbon (for further details, see Materials and Methods). This protocol was developed to avoid the poor/no growth

found on inoculating a non-sugar liquid media directly from a glucose plate (**Table 1**). Using the two-passage protocol for the growth of *SyPy15* yeast on ethanol, the highest growth rate was supported on 3% ethanol. In contrast, no significant differences in growth rates of yeast were detected between being raised on cultures containing 2 or 3% glycerol (**Table 1**). Combining ethanol with galactose or glycerol improved growth compared to ethanol alone (**Table 1**).

Table 1. Growth of *SuPy15* strains supplied with different carbon sources in liquid culture. Culture was carried out according to a two-passage protocol (see Materials and Methods) unless specified as *. Growth rates were determined using the time for the culture to reach x10^7 cells/mL after which the culture was terminated. All values represent the mean ± SE of three biological replicates.

Carbon sources in liquid culture	Cell count (x10^7)		
	24 h	48 h	72 h
Glucose 2%	23.60 ± 1.98	NA	NA
Galactose 2%	19.97 ± 1.58	NA	NA
Ethanol 2%	0.46 ± 0.04	4.35 ± 0.37	NA
Ethanol 3%	0.67 ± 0.06	7.7 ± 0.66	NA
Ethanol 3%*	0.49 ± 0.04	0.59 ± 0.05	0.71 ± 0.07
Ethanol 4%	0.49 ± 0.04	3.7 ± 0.29	7.0 ± 0.55
Ethanol 6%	0.25 ± 0.02	0.22± 0.02	0.33 ± 0.03
Ethanol 9%	0.25 ± 0.02	0.22 ± 0.02	0.16 ± 0.01
Glycerol 2%	1.25 ± 0.10	4.25 ± 0.47	NA
Glycerol 3%	1.18 ± 0.11	5.83 ± 0.43	NA
Glycerol 3%*	0.51 ± 0.04	0.73 ± 0.05	0.81 ± 0.07
3% ethanol / 2% galactose (1:1)	10.23 ± 1.12	NA	NA
3% ethanol / 2% galactose (3:1)	4.2 ± 0.33	NA	NA
3% ethanol / 3% glycerol (1:1)	1.5 ± 0.71	4.33 ± 0.67	NA

*Colonies from glucose plates were directly used to inoculate the liquid culture. NA, not applicable.

The amount of sucrose produced by *SuPy15* cells was affected by the carbon source and whether the media was liquid or solid (**Fig. 4**). While *SuPy15* cells grew on liquid media containing galactose, glycerol or ethanol as well as their combinations (**Table 1**), intracellular sucrose was only detected in liquid culture containing glucose (**Fig. 4A**). However, higher levels of sucrose production occurred when *SuPy15* cells were grown on agar plates containing galactose, ethanol or glycerol (**Fig. 4B**). The highest sucrose production was detected in plate-grown cells with ethanol as the sole carbon source (**Fig. 4B**). The higher sucrose production on plates with non-fermentable carbon sources compared to those in liquid medium suggests that the oxygen supply in liquid medium was not meeting the additional ATP and NADH demands required by glucogenesis to convert ethanol or glycerol to glucose.

A proof of concept for screening sucrose effluxers using *SuPy15*

In order to determine sucrose efflux, *SuPy15* cells were transformed with the sucrose symporter *PvSUT1*, sucrose facilitators *PsSUF4* and *PsSUF1* and the empty vector *pDR196*. The transformants were grown in a liquid glucose medium spiked with [^{14}C]glucose. ^{14}C-sucrose released from cells into the medium was separated from [^{14}C]glucose by thin layer chromatography and its radioactivity levels determined by scintillation counting. This approach was adopted to avoid known glucose interference for enzyme-coupled systems [12, 17], and the weak sucrose signal detected by HPLC due to sample dilution as a result of significantly high ratio of glucose to sucrose [18].

Using this system, we demonstrated that, when *SuPy15* was cultured on a liquid glucose medium, intracellular-synthesized sucrose was preferentially and predictably released from cells transformed with the sucrose facilitators, *PsSUF1* and *PsSUF4*, compared to those transformed with sucrose symporter *PvSUT1* (**Fig. 5**). There was no significant difference in the low levels of sucrose released from *SuPy15* transformed with *PvSUT1* and the vector *pDR196* (**Fig. 5**). The results agreed with the previous findings that both PsSUF1 and PsSUF4, but not PvSUT1, effluxed sucrose when tested in yeast SuSy7 cells pre-loaded with [^{14}C]sucrose [5].

The radioactive TLC approach above offers a sensitive screening of plant sucrose effluxers from any unknown cDNA library clones. The throughput could be greatly increased by adopting an automated workflow for colony picking, microtitre plate-based cultivation and liquid handling [19, 20]. Rapid detection and quantification of radioactive TLC plates can be facilitated by the use of a bioimaging analyzer [21].

While sensitive, we recognize that using large scale radioactive TLC may not offer sufficient capacity for robust high throughput screening of cDNA libraries for sucrose effluxers. This requirement could be achieved by co-culturing *SuPy15* with a sucrose-feeding/sensing bacterium grown on non-sugar carbon sources [22, 23]. In the case of a "yeast-bacteria feeding" system, a sucrose producing yeast *SuPy15* transformed with a plant cDNA library is spread on a SD-plate supplemented with 3% ethanol. Colonies of the yeast transformants are allowed to develop. A small amount of melted agar containing an inoculum of *E. coli* strain, capable of growth on sucrose, is laid over the yeast containing plate (**Fig. 6**). Agar overlay is a well-established technique for screening of DNA libraries typically constructed in lambda phage [24]. The method produces a homogeneous lawn of bacteria within a thin layer of agar across the surface of a plate when the appropriate carbon source is available. Before adopting this technique, the titre of the yeast cDNA library needs to be determined and the dilution of the ttested empirically. Optimized dilution of bacteria in a chemically-defined medium (such as SD or M9 minimal medium without carbon source) is then added to a few ml of soft agar (0.75% agar, as opposed to the usual 1.5% for agar plates) which has been melted and cooled to 42-55°C. The melted agar/bacterial suspension is evenly poured across the top of an agar plate. The idea is that over time, colonies of the *E. coli* strain will develop over the colonies of the yeast strain that efflux sucrose, as the bacteria growing on the agar plates are capable of utilizing sucrose as a carbon

source but not the primary substrate (3% ethanol) supporting growth of the yeast cells. In this context, ethanol could be the best carbon source for yeast growth, and many bacterial strains can not grow on yeast media supplemented with ethanol as the sole carbon source [25]. Colonies of the *E. coli* strain will therefore only develop over colonies of the yeast strain effluxing sucrose. However, a microbial sensitivity test is required to evaluate the distance and concentration of sucrose related to the appearance of the bacteria colonies. This includes a test on the *SuPy15* clones transformed with sucrose transporter genes known to efflux sucrose, and a test on the *SuPy15* clones transformed with empty vector, with/without added sucrose drops. These tests can be used to evaluate the interference of neighbouring colonies. After the first round of screening on a plant cDNA library, the yeast colonies that grow directly, or proximately, under the bacterial colonies are picked, re-plated at appropriate dilutions, and re-screened for their ability to support the formation of bacterial colonies. The process is further repeated to confirm and eventually isolate a single yeast colony that is responsible for developing each targeted bacterial colony. Ultimately, any putatively positive yeast clones must be characterized by the radioactive TLC approach to confirm their ability to release sucrose.

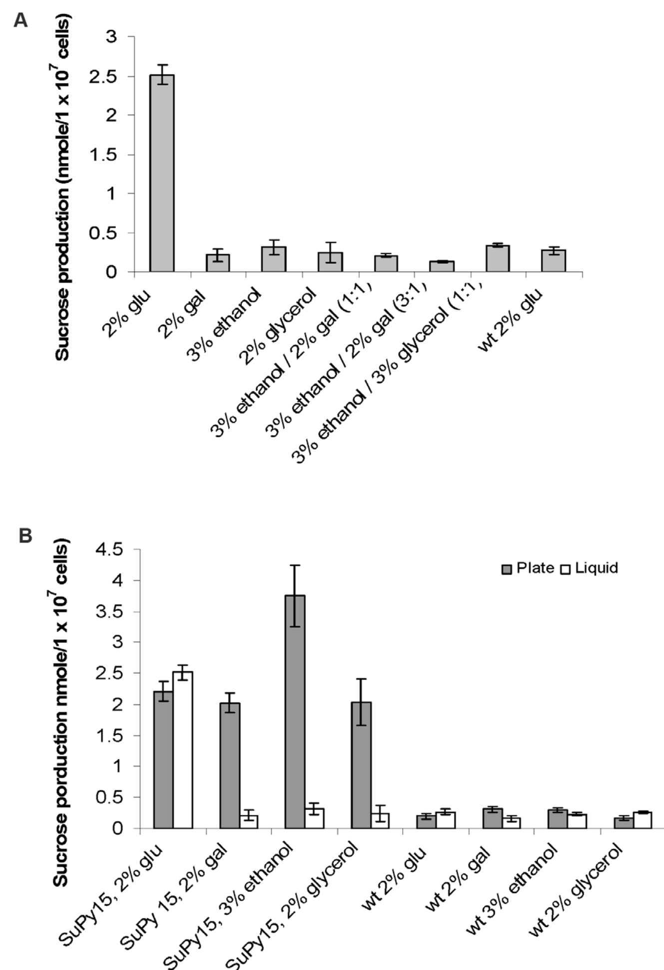

Figure 4. Effect of carbon source on sucrose production in yeast strain *SuPy15* grown in liquid culture (A) or on agar plates (B). For liquid culture, yeast cells were monitored for growth and sampled from mid- to late-log phase (5-7 x 10^7/mL) for sucrose assay. For agar plate culture, yeast was cultured until fully-grown colonies appeared, then scraped off the plate, washed and resuspended in H_2O to a final cell concentration of 5-7 x 10^7 cells /mL for sucrose assay. All values represent the mean ± SE of three biological replicates. Glu: glucose; Gal:Galactose

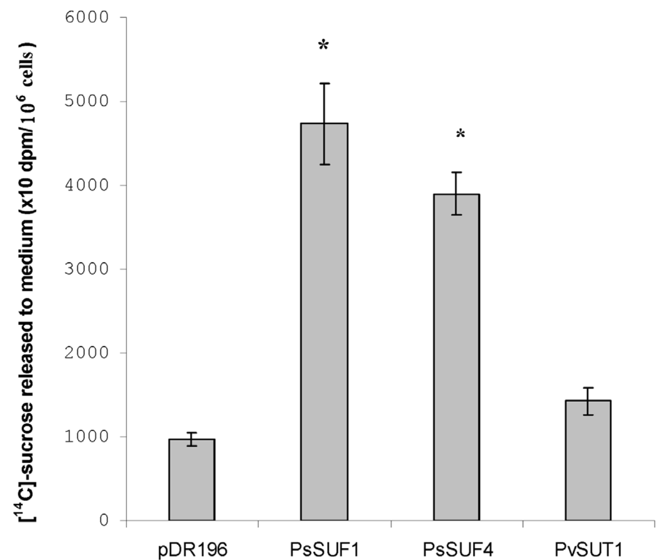

Figure 5. Sucrose efflux from *SuPy15* yeast cultured in 2% glucose containing [^{14}C]glucose (10 µCi/mL) as the carbon source. Supernatants collected after yeast culture were freeze-dried and re-dissolved in 50 uL of ethanol. The extracellular sucrose was separated by spotting 10 µL from each sample on a TLC plate, and its level of radioactivity (dpm) determined by liquid scintillation counting. All values represent the mean ± SE of three biological replicates (*$P < 0.05$ by t-test).

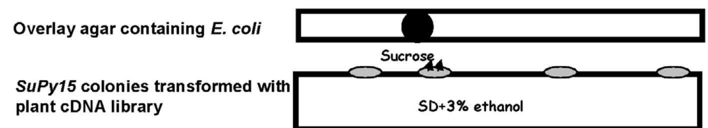

Figure 6. An example of a possible yeast-bacterial feeding assay. A sucrose producing yeast strain, *SuPy15*, transformed with a plant cDNA library is spread on an SD-plate supplemented with 3% ethanol. Colonies of the yeast transformants are developed. The yeast colonies are overlaid by a small amount of melted agar (42-55°C) containing an inoculum of *E. coli* strain in a minimal medium without a carbon source. Colonies of *E. coli* develop over colonies of the yeast strain effluxing sucrose. Yeast colonies that grow directly, or proximal, under the bacterial colonies are picked, re-plated at appropriate dilutions and re-screened. The process is further repeated to eventually isolate a single yeast colony responsible for developing each targeted bacterial colony.

The "yeast-bacteria feeding" system described above could be modified using an agar overlay consisting of the same SD-medium and containing a bacterial sensor strain to detect sucrose [22, 26]. Bacteria expressing a sucrose sensor, e.g. fusion of the bacteria sucrose promoter *scrY* with a *GFP* gene [22] will be visible on top of colonies of the yeast strain effluxing sucrose. In this context, a set of FRET sucrose sensors was developed from a putative *Agrobacterium* sugar-binding protein [27] and these sucrose sensors have been used recently to functionally clone SWEETs in a human cell line transformed with the sucrose sensors [28].

CONCLUSION

A design of, and proof of concept for, a yeast *SuPy15* system to functionally screen sucrose effluxers is described. The *SuPy15* system was shown to support sucrose efflux when transformed with sucrose transporters known to mediate sucrose efflux. The capability of sucrose

accumulation of the strain grown in both sugar and non-sugar medium as sole carbon sources may provide options for high-throughput screening of sucrose released from cDNA library clones.

Acknowledgements

We are indebted to Dr. Mimmi Throne-Holst for helpful discussion on planning the project. Financial support from the Australian Research Council is gratefully acknowledged.

Author contributions

YZ and JWP designed the study, YZ carried out the experiments, YZ, CPLG and JWP interpreted the data and YZ drafted the manuscript with all authors approving the final version.

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