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Identification of Yeast Associated with the Planthopper, *Perkinsiella saccharicida*: Potential Applications for Fiji Leaf Gall Control

Grant L. Hughes · Peter G. Allsopp · Richard I. Webb · Ryuichi Yamada · Inaki Iturbe-Ormaetxe · Stevens M. Brumbley · Scott L. O'Neill

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Abstract Yeasts associate with numerous insects, and they can assist the metabolic processes within their hosts. Two distinct yeasts were identified by PCR within the planthopper *Perkinsiella saccharicida,* the vector of *Fiji disease virus* to sugarcane. The utility of both microbes for potential paratransgenic approaches to control Fiji leaf gall (FLG) was assessed. Phylogenetic analysis showed one of the microbes is related to yeast-like symbionts from the planthoppers: *Laodelphax striatellus*, *Nilaparvata lugens*, and *Sogetella furcifera*. The second yeast was a member of the *Candida* genus, a group that has been identified in beetles and recently described in planthoppers. Microscopy revealed the presence of yeast in the fat body of *P. saccharicida*. The *Candida* yeast was cultured, and transformation was accomplished by electroporation of *Candida albicans* codon optimized plasmids, designed to integrate into the genome via homologous recombination. Transgenic lines conferred resistance to the antibiotic nourseothricin and expression of green fluorescent protein was observed in a proportion of the yeast cells. Stably transformed yeast lines could not be isolated as the integrative plasmids presumably replicated within the yeast without integration into the genome. If stable transformation can be achieved, then this yeast may be useful as an agent for a paratransgenic control of FLG.

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G. L. Hughes · R. Yamada · I. Iturbe-Ormaetxe · S. L. O'Neill
School of Biological Sciences, The University of Queensland, Brisbane, QLD 4072, Australia

Present Address:
G. L. Hughes
The W. Harry Feinstone Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health and Malaria Research Institute, Johns Hopkins University, 615 North Wolfe Street, Baltimore, MD 21205, USA

P. G. Allsopp
BSES Limited, P.O. Box 86, Indooroopilly, QLD 4068, Australia

R. I. Webb
Centre for Microscopy and Microanalysis, The University of Queensland, Brisbane, QLD 4072, Australia

Present Address:
R. Yamada
Department of Metabolism & Aging, The Scripps Research Institute, 130 Scripps Way, Jupiter, FL 33458, USA

Present Address:
I. Iturbe-Ormaetxe · S. L. O'Neill (✉)
School of Biological Sciences, Monash University, Melbourne, VIC 3800, Australia
e-mail: scott.oneill@monash.edu

S. M. Brumbley
Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, Brisbane, QLD 4072, Australia

Present Address:
S. M. Brumbley
Department of Biological Sciences, University of North Texas, Denton, TX 76203-5017, USA

Introduction

Arthropod-borne diseases cause major impacts in terms of morbidity and mortality to humans, and have a devastating impact on livestock and agriculture. Paratransgenesis, a strategy that uses transgenic microbes associated with insects to express molecules that interfere with pathogen transmission, is an approach to decrease the burden of these diseases [6]. Microbes, which commonly associate with insects, offer an alternative host for transformation, and alleviate the need for genetic manipulation of the vector. Ideal candidates for paratransgenesis are microbes that maintain an intimate association with the host to facilitate the transmission of transgenic symbionts through the insect population [5].

Fiji leaf gall (FLG) is a debilitating viral disease of sugarcane. *Perkinsiella saccharicida* is the vector of Fiji disease virus (FDV) within Australia, the causal agent of FLG to sugarcane [18, 31]. In the planthopper, the virus is transmitted persistently to sugarcane, whereby the virus replicates in the insect and once the host is infective, it is viruliferous for life [44]. For successful transmission, the virus needs to be acquired by the insect from a diseased plant, replicate in the host insect, translocate across membrane barriers within the insect to ultimately reach the salivary glands to be expelled to a susceptible plant [2]. Each of these stages is a potential bottleneck where virus transmission can be disrupted.

Delphacid planthoppers are known to harbor endosymbiotic bacteria and yeasts [25, 35, 48]. *Laodelphax striatellus* and *Nilaparvata lugens* possess yeasts that are classified within the *Candida* genus [3, 16]. In other insects, *Candida* yeasts have been identified in the gut, which are presumably involved in nutrient provisioning and are vertically transmitted to their progeny by coating the egg shell [46, 51]. Other yeast-like symbionts (YLS) in planthoppers reside in mycetocytes within the insect, and are maternally transferred [32, 34, 48]. These YLS are not free-living in nature and are impervious to culture, most probably because of the unique and specific environment provided by the insect host.

While several paratransgenic approaches have been proposed using bacteria [1, 17, 19], the use of yeast as a vehicle for paratransgenesis is relatively new. Recently, yeasts have been described in *Aedes* and *Anopheles* mosquitoes and have been suggested as a candidate microbe for paratransgenesis [39, 40]. The localization of these microbes in tissues, proximal to pathogens, holds promise for their use in paratransgenesis if suitable transformation techniques are devised for these yeasts. In this study we characterized the yeast in *P. saccharicida*, the vector of FDV within Australia. We then assessed the suitability of *Candida* yeast associated with *P. saccharicida* for a paratransgenic approach to control FLG and to describe initial transformation attempts.

Materials and Methods

Amplification and Phylogenetics of YLS from Planthoppers

DNA was extracted from planthoppers surface sterilized with 10% bleach collected at BSES research station (Woodford, Queensland, Australia) [29]. Individual planthoppers, dissected ovaries, eggs, or abdomen tissues were homogenized followed by DNA extraction using the Puregene cell and tissue DNA isolation kit (Gentra Systems, MN) following the manufactures protocol. Universal fungal primers were used for amplifying a region of the 18S rDNA [53], D1/D2 region of the 26S rDNA [12] (Supplementary table 1). As these primers also amplified insect host DNA, specific forward primers (YLS-18S-F and YLS-26S-F) were designed which only amplified yeast when used in conjunction with the universal reverse primer (Supplementary table 1). PCR was performed on *P. saccharicida* DNA using *Taq* polymerase (NEB) according to the manufacturer's guidelines. PCR products were TA cloned into a pGEM-Teasy vector (Promega) and Sanger sequenced. A restriction fragment length polymorphism (RFLP) analysis using *Pst*I or *Aat*II, which uniquely cut the D1/D2 region of either yeast, was used for ascertaining the prevalence of the two yeast species in the host. Partial rDNA sequences were aligned in ClustalX [50] with closely related taxa. After estimation of an evolutionary model using Modeltest [38], Bayesian inference of phylogeny was constructed using MrBayes 3.1.2 [23, 41]. Phylogenic trees were edited and rooted in Treeview 1.6.6 [37].

Culturing Yeast-like Symbionts

Yeasts were isolated from surface-sterilized [29] whole insects and unsterilized eggs of *P. saccharicida,* crushed in 0.7% saline solution and plated onto acidified Difco yeast malt (BD, Sparks, MD) agar plates (YM plates), and then incubated at 30°C until they attained yeast colony growth (3–4 days). Eggs were obtained by dissecting sugarcane leaves that planthoppers had been reared upon [24]. Individual colonies were then sub-cultured on YM plates to obtain pure cultures and DNA was extracted from single colonies [22]. The yeast species was confirmed by sequencing the 26S rDNA region and a segment of the ITS region [53] (Supplementary table 1).

Antibiotic Sensitivity Assay

Five antibiotic/antifungal agents, *p*-fluoro-DL phenalinine (PFP) (1–5 mg/ml) [11], benomyl (1–5 µl/ml) [10], nourseothricin (Nat) (50–150 µg/ml) [20], nystatin (5–20 µg/ml), and kanamycin (50–200 µg/ml) were assayed to determine which agent to be used in transformation procedures. A liquid culture of yeast was spread onto plates containing the specific antibiotic with each plate receiving the same concentration of starter culture. The plates were incubated at 30°C, and the growth of yeast was recorded on each plate daily.

Construction of Plasmids

DNA manipulation and transformation of *E. coli* were carried out according to Sambrook et al. [42]. The *SAT* gene (conferring Nat resistance) driven by the *C. albicans* actin promoter and the *C. albicans* URA3 terminator was amplified from pACT1G1 [30] (Fig. 1) using the primers CaSAT-F and CaSAT-R (Supplementary table 1), and subcloned into pGEM-Teasy vector (Promega). The URA3 selection gene from the plasmid pACT-GFP [4] was replaced with the *SAT1* resistance gene via *Not*I and *Spe*I digestion, and subsequent cloning producing the vector pACT-GFP::SAT (Fig. 1). To construct an integrative plasmid, which had native sequence from the *Candida* sp. from *P. saccharicida* for homologous recombination, the *Candida RIB7* gene was transferred into pACT-GFP::SAT replacing *Ca*RSP1 gene. The *RIB7* was amplified using primers RIB7-BamHI-F and RIB7-BamHI-R, thereby introducing *Bam*HI sites at the ends [9] (Supplementary table 1). The PCR product was TA cloned into pGEM-Teasy vector (Promega) and sequenced. This plasmid was subsequently digested with *BamH*I, and the insert was blunt end ligated into pACT-GFP::SAT digested with *BamHI–Nhe*I, after treatment of both the insert and vector with T4 DNA polymerase in the presence of dNTPs, to create the plasmid pACT-GFP::SAT-RIB (Fig. 1). For transformation with this vector, riboflavin (200 mg/L) was supplemented to the media for yeast growth. To create a linearized fragment, plasmids pACT-GFP::SAT and pACT-GFP::SAT-RIB were digested with *Stu*I and *Bsa*BI, respectively. This was resolved on a 1% agarose gel and DNA extracted for transformation experiments using a gel extraction kit (Qiagen). A fragment containing the resistance gene and GFP flanked by segments of the *C. albicans* actin gene was excised from pACT1G1 with *Xho*I and *Sac*II digestion. Uncut pACT-GFP::SAT and pACT1G1 were also used to transform yeast.

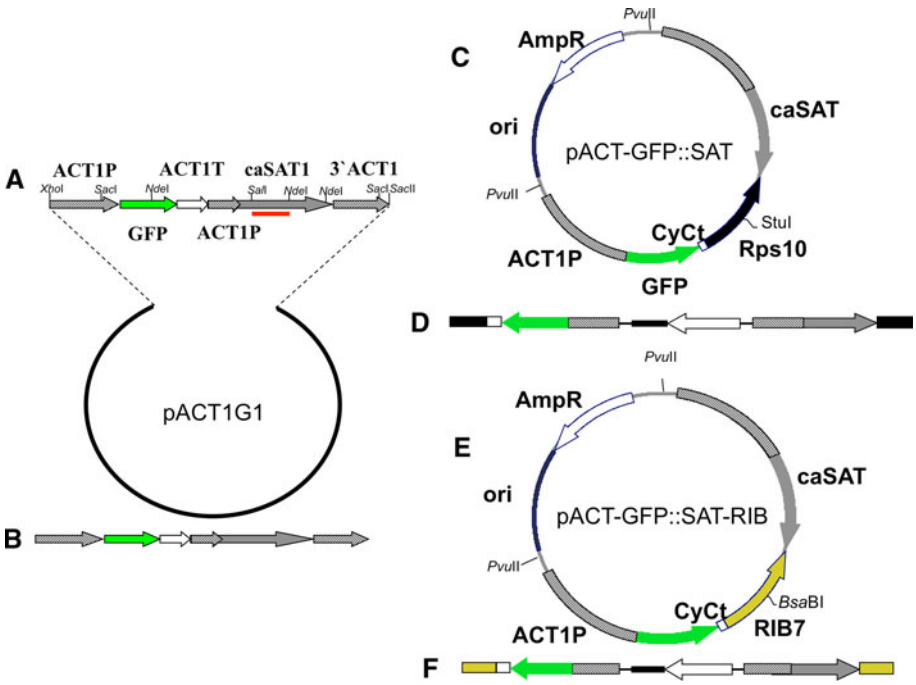

Fig. 1 Map of plasmids used for transformation of *Candida* yeast. Plasmid pACT1G1 (**a**) was digested using *Xho*I and *Sac*II to produce a DNA fragment with GFP and the *Ca*SAT resistance gene flanked by the *Ca*Actin promoter and 3′region (**b**). The plasmid pACT-GFP::SAT (**c**) was linearized by a *Stu*I digestion providing a DNA fragment with the *Ca*Rps10 gene fragments flanking the internal sequence of the plasmid (**d**) The plasmid pACT-GFP::SAT-RIB has the native *Candida* yeast *RIB7* sequence (**e**) which has replaced the *Ca*Rps sequence from pACT-GFP::SAT. The pACT-GFP::SAT-RIB plasmid is linearized with a *Bsa*BI digestion that cuts the *RIB7* gene (**f**). The segment of plasmid that was used for probing DNA in Southern hybridization (Fig. 5) is indicated with a *red line* in the pACT1G1 plasmid (*Sal*I/*Nde*I digestion) (**a**). Each plasmid has the *SAT* gene which provides resistance to Nat; therefore, this probe will hybridize to all constructs

Yeast Transformation

Yeasts were transformed via lithium acetate pretreatment followed by electroporation [54]. DNA was extracted from transgenic and wild-type yeast lines as previously described. For the pACT1G1 lines, primers up- and down-stream (Cg-Actin-150, Cg-Actin + 2500) of the putative insertion site within the actin gene paired with a primer binding within the transgene (GFPUVPR, SAT-F) were used for screening for integration (Supplementary table 1).

Southern Hybridization Analysis

DNA was resolved by gel electrophoresis and capillary transferred to Hybond-XL nylon membranes (Amersham) [42]. For each treatment, 10 µg of DNA (wild type or transgenic lines with or without selection) was digested with restriction enzymes (pACT1G1 with *Sac*I, *EcoR*I, and *BamH*I; pACT-GFP::SAT with *Pvu*II, *EcoR*V, and *Nsi*I; pACT-GFP::SAT-RIB with *Pvu*II, *Nsi*I and *Nhe*I). A *Sal*I/*Nde*I fragment encoding the SAT gene was digested from pACT1G1 and was employed to probe the membrane. Probe sequence was also hybridized to the membrane as an internal positive control. The probe was labeled with ^{32}P using a random labeling kit (Roche), and the membrane was exposed to a Molecular Dynamics storage phosphor screen (Amersham). The signal was measured using a Storm 830 phosphoimager (Amersham).

Plasmid Rescue from Transgenic Yeast and Stability Assay

Plasmids were isolated from transgenic yeast cultures using a mini-prep kit (Qiagen) after treating yeast with zymolase (25 µg/µl zymolyase in 10 mM Na_2PO_4 and 50% glycerol). Plasmids or total genomic DNA was transformed into chemically competent *E. coli* DH5α by heat shock using standard methods [42] and colonies grown on selective media. Rescued plasmids were compared in size with the initial plasmids used for transformation by restriction digestion. The stability of plasmids in transgenic yeast was measured by growth at 30°C under non-selective condition in YM liquid media for 5 h, followed by inoculation (100 µl) onto selective or non-selective plates. After 4 days, the number of colonies on antibiotic or non-antibiotic media was counted to determine plasmid stability [26].

Microscopy

Planthopper abdomens or samples from cultured YLS were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer and subsequently processed using a Pelco Biowave microwave oven using a protocol similar to Wendt et al. [52] and embedded in Spurr's resin. Ultrathin sections were viewed using a JEOL 1010 transmission electron microscope (TEM), while semi-thin sections were observed using a Zeiss microscope (Zeiss). Light and fluorescent microscopies were conducted using a Zeiss microscope (Zeiss). Images were captured by a digital camera using AxioCam software.

Results

Phylogeny and Prevalence of Yeast in Planthoppers

The 18S and 26S D1/D2 ribosomal regions were amplified from both the yeasts, while the ITS sequence was obtained from the cultured *Candida* yeast (Genbank accessions: EU400269-EU400273). Phylogenetic analysis of the D1/D2 region of the 26S rDNA region (Fig. 2) revealed the presence of two distinct YLS in *P. saccharicida*. One yeast (herein designated *P. saccharicida*-YLS (Ps-YLS)) had similarity to YLSs from *L. striatellus, N. lugens* and *Sogetella furcifera* [48], while the *Candida sp*. grouped with other *Candida* and *Pichia* yeasts that associate with basidiocarp-feeding beetles [45]. The 18S (data not shown) and 26S rDNA phylogentic trees were congruent and comparable to previously published yeast phylogenetic trees [46–48]. RFLP analysis was completed on yeast from single planthoppers from different geographic regions. Of the 16 planthoppers tested from the Woodford region, only one planthopper was not positive for either yeast. All other planthoppers sampled were found to be co-infected with both yeast species ($n = 45$). Additionally, consensus sequences for each species were identical within and between geographic regions for both yeasts.

Localization of Yeast Within the Planthopper

PCR analysis identified *Candida* and Ps-YLS in abdomen tissue of the insect. In addition, ribosomal RNA genes of the Ps-YLS were amplified from dissected ovaries and eggs from female planthoppers. Light microscopy showed the microbes were ubiquitous in adult planthoppers and distributed throughout the abdomen of the insect (Fig. 3a–c). TEM further elucidated the location of the symbiont around fat globules in nymphs (Fig. 3d, e, g). Microscopy found that no yeast was in the gut of the insect. Budding in the yeast within the insect appeared to be more toward the polar regions (Fig. 3h), while axial budding could be observed from the cultured *Candida sp.*, although there was evidence that previous buddings (budding scars) were in the polar region of these yeast (Fig. 3i). Using TEM, it was not possible to differentiate between the two symbionts in the insect.

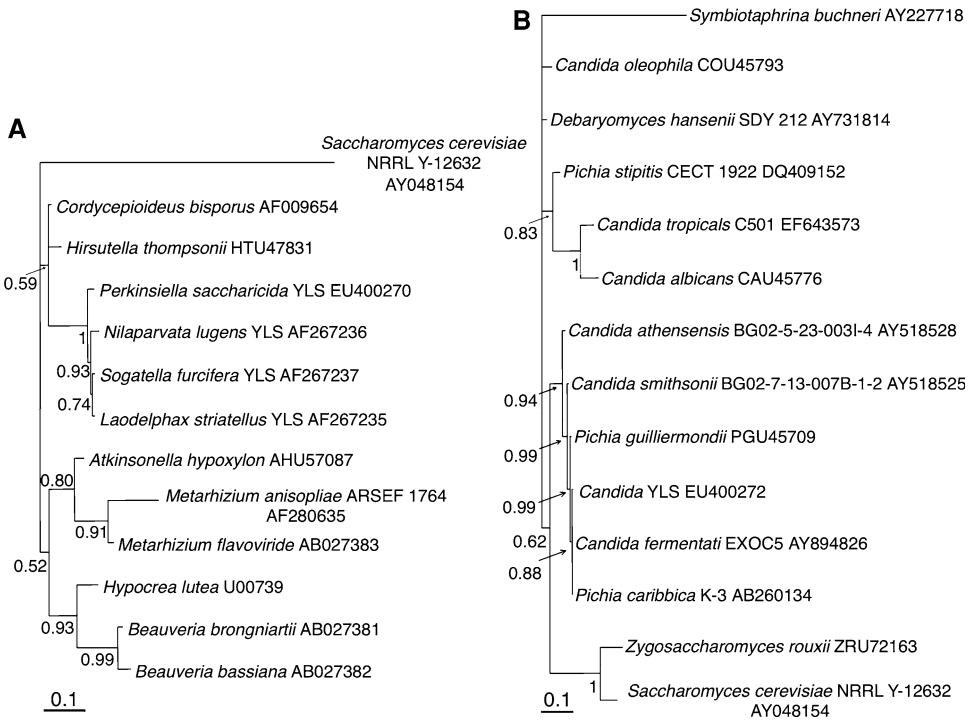

Fig. 2 Bayesian phylogenetic analysis of the Ps-YLS (**a**) and the *Candida* yeast (**b**) using the D1/D2 26S rDNA gene. Markov chain Monte Carlo analysis was run in duplicate (4 chains each) with default parameters for one million generations with samples collected every 100 generations in MrBayes. Bayesian posterior probabilities were calculated by computing a 50% majority rule consensus of the trees, remaining from the duplicate runs after discarding the burn-in that represented 25% of trees. *Numbers* indicated on tree branches represent posterior probabilities confidence values, while the *scale bar* represents the number of substitutions per site. Genebank accession numbers are listed beside each taxa

Culturing of *Candida* Yeast

The *Candida* yeast, identified in the PCR assays from *P. saccharicida*, was cultured axenically using the methods previously devised for culturing of yeast symbionts of insects [46]. Yeast were cultured from surface sterilized crushed planthoppers and unsterilized eggs. In addition, saline solution used for washing eggs was used as inoculum to culture yeast. Sequences of the 26S D1/D2 region of the rDNA from the cultured yeast were identical to sequence obtained from the *Candida* sp. identified in the planthopper, indicating that the yeast cultured was from the planthopper and not an environmental contaminant.

Transformation

Antibiotic sensitivity assays were undertaken to identify a suitable selective agent to be used for transformation experiments. PFP and Nat successfully inhibited yeast growth at all tested concentrations, and Nat was subsequently used for transformation experiments. The *Candida sp.* was transformed with the linearized integrative plasmids pACT-GFP::SAT and pACT-GFP::SAT-RIB, and the excised segment of pACT1G1, and after transformation, colonies grew on media containing 100 μg/ml Nat. However, only a small proportion of cells expressed green fluorescent protein (GFP). Wild-type controls displayed little or no autofluorescence when compared with their transgenic counterparts (Fig. 4). After sub-culturing from plates containing antibiotic, GFP expression was analyzed in transgenic yeast from cultures that had been grown with and without antibiotic selection. The number of cells expressing GFP was dramatically reduced when grown without antibiotic compared with yeast grown on Nat media (Fig. 4). GPF expression in yeast lines was heterogenous, with fluorescence being only observed in a proportion of yeast cells in the transgenic lines. This was constant throughout all transgenic lines when a selection pressure was applied, with approximately 10% of cells exhibiting fluorescence (Fig. 4). Expression was lost in all lines when the yeast was grown continuously on non-selective media. Moreover, an active selection pressure was required to sustain the plasmid in yeast population in all transgenic lines. Without antibiotic, the percentage of transgenic yeast from one subculturing dropped to around 1.5% for lines transformed with the pACT1G1 plasmid and 0.5% from of lines created with pACT-GFP::SAT the plasmid, which clearly demonstrate that active selection is required to keep these plasmids within the yeast population.

Verification of Insertion

Southern hybridization analysis revealed that lines transformed using the linearized plasmid designed to integrate into the genome and control lines with uncut plasmids had similar sized DNA fragments that hybridized to the probe, suggesting that intact plasmids were replicating within transgenic yeast lines (Fig. 5). Similar banding patterns were recorded for lines grown on selective and non-

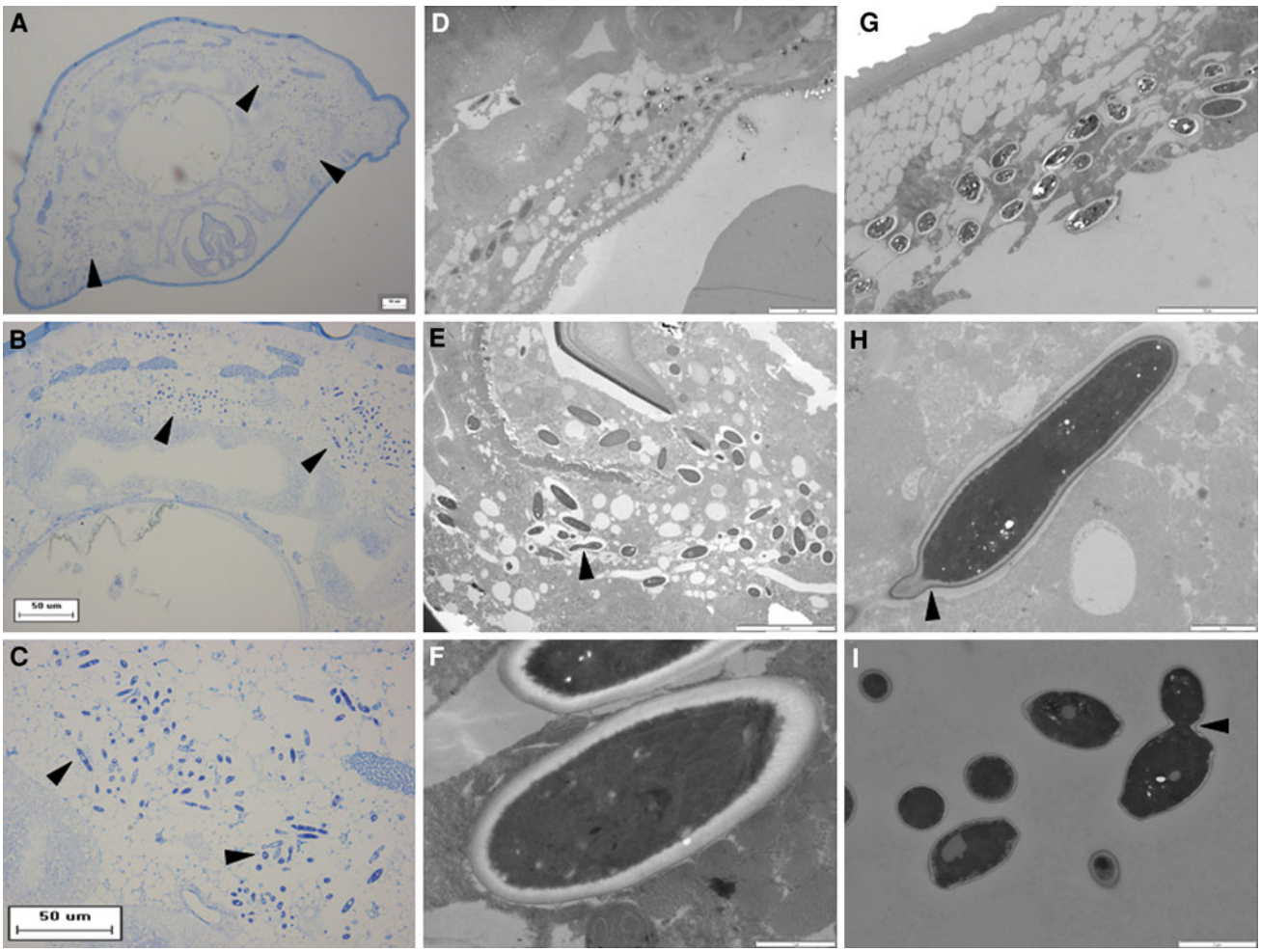

Fig. 3 Light microscopy (**a**–**c**) and transmission electron microscopy (**d**–**i**) of yeast in *P. saccharicida*. In a transverse section yeast can be seen scattered though the insect tissue (50× (**a**), 200× (**b**), and 400× (**c**)). *Arrows* indicate yeast. Scale bar on all images **a**–**c** is 50 μm. TEM of yeast within planthoppers (**a**–**h**) and from pure culture (**i**). Yeast is residing within the fat body below the cuticle (**d**, **e**, **g**). Yeast is seen undergoing budding (*arrowhead*) within the insect (**e**, **h**), and within culture (**I**). The *scale bar* represents 20 μm for images **d**, **e** and **g**, 2 μm for images **h** and **i,** and 10 and 1 μm for images **f**

selective media, although the signal from the latter was faint. Moreover, PCR amplification across the supposed integration point was unsuccessful (data not shown). In concordance with the Southern analysis data suggesting replication of plasmids within transgenic lines, whole plasmids were rescued from transgenic sub-cultured pACT1G1 lines by electroporation of DNA extracted from yeast into *E. coli* cells or by isolating the plasmid from transgenic yeast cultures.

Discussion

Planthoppers are known to harbor YLS [32, 34, 48]. However, until recently, only *Ascomycetes* YLSs within planthoppers had been characterized at the molecular level [48], despite the observation of morphologically diverse yeast from these insects [27, 33]. In this study, however, we find that *P. saccharicida* is infected by two distinct yeast species, which adds to the growing body of evidence regarding the diversity of yeast within planthoppers. Phylogenetically, both symbionts cluster with previously known yeasts that associate with insects, with one yeast grouping monophyletically with *Ascomycetes* YLSs from the planthoppers *N. lugens*, *S. furcifera* and *L. striatellus* [48]. The other *Candida* yeast is similar to yeast associated with basidiocarp feeding beetles [45] but has recently been found in planthoppers [3, 16].

PCR analysis identified both microbes in multiple planthoppers from geographically distinct regions suggesting that these species are commonly associated with the insect. Within the insect, PCR localized both yeasts to the abdomen of the planthopper, while culturing techniques isolated the *Candida* sp. from whole planthoppers, and the

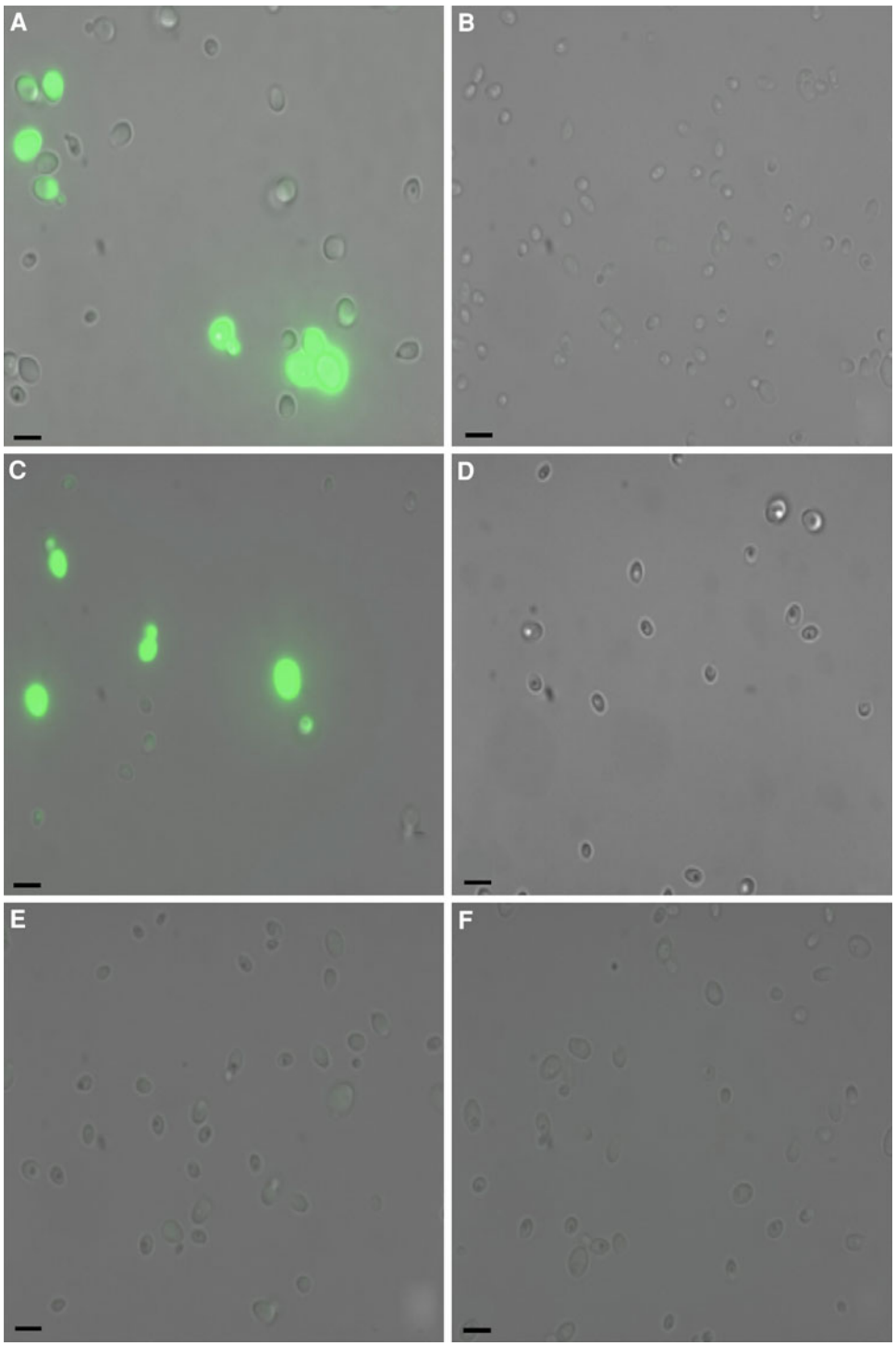

Fig. 4 GFP expression in *Candida* yeast transformed with linearized pACT-GFP::SAT. All transgenic lines had similar GFP expression profiles. Images are at 1,000× magnification. Fluorescent and differential interference contrast (DIC) microscopy-captured images were merged to create a single image. Single yeast colonies were grown under Nat antibiotic selection (**a**) or no selection (**b**). Single colonies from both plates were picked and grown with or without selection. Colonies picked from the Nat antibiotic plate (**a**) were subjected to a second round of Nat antibiotic selection (**c**) or no selection (**d**), while colonies picked from the plate under no selection (**b**) were grown on Nat antibiotic selection (no growth recorded) or no selection (**e**). Wild-type yeasts exhibit no autofluorescence (**f**) *Bar* indicates 5 μm

surface of the eggs. Also, the Ps-YLS was identified by PCR of dissected ovaries. Microscopy further elucidated that yeast was present in the fat body of nymph and adult planthoppers, similar to the observations in previous studies in *L. striatellus* [34] and *N. lugens* [13]. Recently, Dong and colleagues [16] identified both *Ascomycetes* yeast and yeast with similarity to *Pichia guilliermondii* (*Candida guilliermondii* is the anomorphic form of *P. guilliermondii*) in the fat bodies of *N. lugens*. Given the similarity between both the host planthoppers and the yeast species harbored within, it is tempting to speculate that yeasts identified in the fat bodies of *P. saccharicida* by microscopy are both Ps-YLS and *Candida* species. In *N. lugens*, YLSs residing in the fat body migrate through the hemolymph to

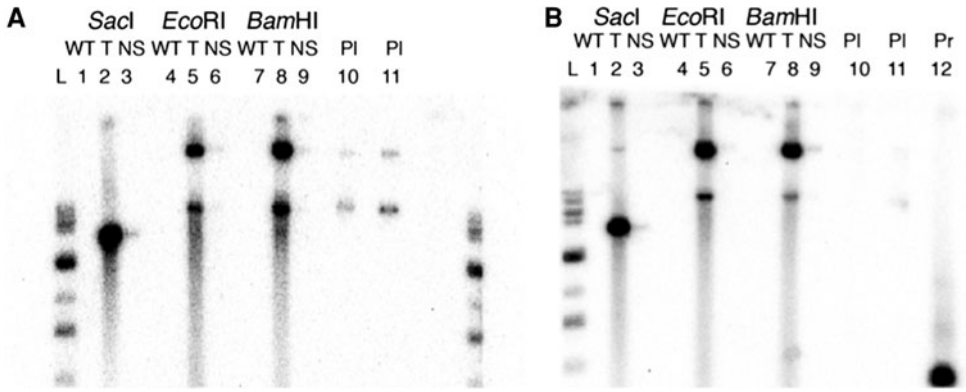

Fig. 5 Southern hybridization analysis of two transgenic lines generated with either the pACT1G1 fragment cut with *Xho*I/*Sac*II (**a**), or pACT1G1 plasmid undigested (**b**). For each Southern hybridization DNA extracted from wild-type lines (WT), transgenic lines (T) or transgenic lines grown with no selection (NS). DNA was digested with a restriction enzyme before capillary transfer; DNA was either digested with an enzyme that cut within the plasmid (*lanes 1–3*) or an enzyme that did not cut the plasmid (*lanes 4–9*). Lanes marked Pl are uncut control plasmid, and the lane marked Pr is the probe (*lane 12*). For transgenic lines, where the enzyme does not cut the plasmid (*lanes 5*, *6*, *8*, and *9*) the probe hybridized to DNA that had the same molecular weight as did the uncut control plasmid (*lanes 10–11*), suggesting that intact plasmids had replicated within transgenic yeast lines. All the membranes were probed with a segment of the *SAT* resistance gene obtained by *Sal*I/*Nde*I digestion of pACT1G1 randomly labeled with ^{32}P (Fig. 1)

ultimately reach the oocytes and are transovarially transmitted to the progeny [13]. A similar mechanism may be employed here, but as the *Candida* yeasts were cultured from the inoculum of washed eggs, the *Candida* yeast may be smeared onto the egg in a similar fashion to yeast transmission in basidiocarp-feeding beetles [51].

Paratransgenesis utilizes microbes associated with insects to manipulate the vector competence of the host. To date, most paratransgenic approaches have employed bacteria associated with insects; however, recently, yeasts have been proposed as candidate microbes for this approach [39, 40]. The symbiotic nature of both YLSs in planthoppers, and *Candida* yeast in other insects has been elucidated [43, 51]. Although not tested empirically here, it could also be conjectured based on phylogeny that these yeasts have similar biological roles in *Perkinsiella* planthoppers. Regardless of the nature of the symbiosis, the high prevalence of these microbes in *Perkinsiella* populations suggests that these yeasts could be applied for paratransgenesis. As the *Candida* yeast was amenable to cultured, we investigated transformation of this microbe.

Sequencing of the D1/D2 regions of the 26S rDNA region of the *Candida sp.* confirmed the cultured yeast was isolated from the planthopper. Transformation of yeast was undertaken using integrative plasmids of *Candida albicans* (pACT1G1 and pACT-GFP::SAT), which incorporated into the actin or *RPS10* gene via homologous recombination. A third plasmid, pACT-GFP::SAT-RIB, was created, which used the native sequence of the *RIB7* gene from the *Candida* yeast from planthoppers for homologous recombination to circumvent the possibility that a lack of sequence identity was interfering with the integration process. Similar integrative transformation techniques have been applied successfully to close relatives of the *Candida* yeast from *P. saccharicida*, including numerous *Candida* spp. [14, 15, 28] and *Pichia* spp. [21, 36].

The expression of the GFP and growth on antibiotic media show that *C. albicans* promoters and genes with optimized codon usage (CUG codons coding for leucine) are functional in *Candida* yeast from *P. saccharicida*. Approximately 10% of cells expressed GFP when grown under selective conditions, which was consistent throughout all transgenic lines. Temporal and developmental variation in cell cycle may influence GFP expression. This may also explain why yeast populations under antibiotic selection were polymorphic for GFP expressing and non-expressing cells. The inability to grow yeast on antibiotic plates after subculture on plates without antibiotic indicates the plasmid is lost from the transgenic yeast when grown without selection.

Taken together, the combined GFP expression data Southern hybridization analysis, low plasmid stability, and the ability to rescue whole plasmids from transgenic yeast DNA are indicative that transformation was replicative and not integrative. The lack of a stable line, when grown without the presence of selection, prevented the re-infection of planthoppers with transgenic yeast, as feeding antibiotics to the insect resulted in mortality (data not shown). It is feasible that when resolving linearized plasmids or fragments of plasmids that were used for transformation, a low concentration of uncut plasmid was also isolated, which was subsequently transformed to the yeast and replicated by an unknown mechanism. There are multiple examples whereby integrative transformation attempts have led to unexpected plasmid replication occurring or capture of chromosomal fragment that allows plasmid replication in diverse yeast species [7, 8, 49].

This study found two distinct yeasts residing within *Perkensialla* planthoppers. The ability to culture and to transform the *Candida* yeast, although in a replicative fashion, suggests that this microbe may be a candidate for paratransgenesis to control FLG in *P. saccharicida* and warrants further investigation. Yeasts offer a new category of microbes to be used for paratransgenesis and compared with bacteria, yeast present new opportunities and challenges for the successful implementation of paratransgenesis.

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