Gateway Vectors for Efficient Artificial Gene Assembly In Vitro and Expression in Yeast Saccharomyces cerevisiae

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Abstract

Construction of synthetic genetic networks requires the assembly of DNA fragments encoding functional biological parts in a defined order. Yet this may become a time-consuming procedure. To address this technical bottleneck, we have created a series of Gateway shuttle vectors and an integration vector, which facilitate the assembly of artificial genes and their expression in the budding yeast Saccharomyces cerevisiae. Our method enables the rapid construction of an artificial gene from a promoter and an open reading frame (ORF) cassette by one-step recombination reaction in vitro. Furthermore, the plasmid thus created can readily be introduced into yeast cells to test the assembled gene’s functionality. As flexible regulatory components of a synthetic genetic network, we also created new versions of the tetracycline-regulated transactivators tTA and rtTA by fusing them to the auxin-inducible degron (AID). Using our gene assembly approach, we made yeast expression vectors of these engineered transactivators, AIDtTA and AIDrtTA and then tested their functions in yeast. We showed that these factors can be regulated by doxycycline and degraded rapidly after addition of auxin to the medium. Taken together, the method for combinatorial gene assembly described here is versatile and would be a valuable tool for yeast synthetic biology.

Introduction

Recent progress in synthetic biology has made it possible to engineer gene regulatory networks with predictable behaviors [1]. Construction of de novo gene networks requires efficient assembly of DNA sequences including promoters and ORFs encoding proteins with defined functions. Yet such DNA assemblies are often time-consuming, typically involving ad hoc procedures such as restriction digestions and DNA ligations. An alternative method for gene assembly is the multistep Gateway recombination cloning [2]. This system enables rapid and highly efficient construction of an expression vector containing multiple DNA fragments in vitro from the so-called Entry clones and a Destination vector. A recent study applied this cloning technology successfully to the yeast one-hybrid system [3]. In this system a library of DNA bait sequences and ORFs encoding the reporters (His3 and β-galactosidase) were assembled in a one-step Gateway recombination reaction. The cloned plasmids were then integrated at the HIS3 and URA3 loci for screening [3–5]. However, the Gateway vectors for the one-hybrid system were not designed as versatile plasmid vectors for more general use to build artificial genes and introduce them into yeast. Such vectors should facilitate the rapid characterization of new promoter-ORF combinations prior to the synthesis of an artificial genetic network in yeast.

In an effort to aid gene network construction in budding yeast Saccharomyces cerevisiae, we have adopted an approach similar to the one-hybrid system and created Gateway Destination vectors. These new Destination vectors are based upon the pRS series of yeast centromere plasmids [6] and an integration vector [7]. In addition, we have constructed a set of Entry clones harboring promoters and ORFs. An artificial gene from a promoter and an ORF Entry clones can be assembled in a Destination vector by one-step recombination reaction in vitro.

To increase the flexibility of regulation of synthetic genetic networks in yeast, we engineered two transcriptional transactivators, AIDtTA and AIDrtTA. Each of these are a fusion of well-characterized functional protein domains, the auxin-inducible degron (AID) [8] and the tetracycline-regulated transactivator (tTA and rtTA) [9]. tTA activity is downregulated in the presence of tetracycline antibiotics such as doxycycline, while rtTA is activated by the antibiotic. In the AID system, a plant hormone auxin binds to Tir1, an F-box protein, which in turn induces rapid degradation of the target protein fused to AID [8]. Using our Gateway vectors and the recombination cloning method, we constructed expression vectors of AIDtTA and AIDrtTA and tested their functions in yeast. We also constructed a destabilized version of Venus (enhanced YFP) ORF, yEvenus-Cln2PEST-NLS, to be expressed as a fluorescent reporter protein. Taken together, these resources provide a technological platform to assemble and test a new promoter-ORF combination efficiently in yeast.

Results

Construction of Yeast Gateway Vectors for One-step Gene Assembly

To construct Gateway Destination vectors for assembling a promoter and an ORF, we cloned the DNA sequences for site-specific recombination and terminators for gene expression into
the pRS series of yeast centromere plasmids [6] and an integration vector [7]. Table 1 is the list of Destination vectors created in this study (see Materials and Methods for details). We also made a collection of promoter and ORF Entry clones by Gateway BP recombination reactions (Table 2). The constructed Destination vectors (Fig. 1) can be recombined with a promoter Entry clone and an open reading frame (ORF) Entry clone in a one-step Gateway LR reaction in vitro (Fig. 2). These promoters and ORFs were cloned in the Gateway Donor vectors in a specific direction so that assembled genes in expression vectors are always in the same orientation relative to the recombination sites, in the order of attB2-[promoter]-attB3-[ORF]-attB1-[TEF or CYC1 terminator]. The attB5 sequence (5’-CAACCTTTTAGTACAAAGGTTG-3’) has no noticeable side effect on the gene expression from vectors so far created by this method. A recent study has also applied the Gateway system successfully for a yeast one-hybrid system, in which a single attB1 recombination sequence is created between a promoter and an ORF as a by-product of the recombination event [3–5]. The attB5 ‘scar’ left behind by LR reactions may therefore be unlikely to interfere with the assembled gene’s expression.

One of the Destination vector we have created (pDEST375) allows integration of the artificial gene made by a Gateway recombination reaction at the MET15 locus (Fig. 3). This construct is based on the integration vector pIS375, which allows a single-copy integration of the gene at the MET15 locus [7]. The integrated constructs are highly stable because a duplicated copy of MET15 and the flanking plasmid sequences including the URA3 marker gene are removed by homologous recombination [7] (Fig. 3). It enables a recycling of URA3 marker gene for subsequent gene integrations at other loci in the genome. pDEST375 was used for the integration of TEF-AIDrtTA gene (see below and Materials and Methods for details). For the detailed description of this class of integration/disintegration vectors, see Sadowski et al. [7].

We have performed a number of Gateway LR reactions using these plasmids with various combinations of promoters and ORFs to create Yeast/E. coli shuttle vectors. The vectors thus created for this study are listed in Table 3. An example of the expression vectors with various combinations of promoters and ORFs (Table 2) are transformed with a vector harboring ADH1-AIDrtTA (pDHM19). The transformed cells were grown in liquid medium and treated with 1-naphthaleneacetic acid (NAA, a synthetic analog of auxin) or left untreated. Cells were then harvested 0.5, 3 and 4 hours after the addition of NAA, and the expression of AIDrtTA was examined by Western blotting using a monoclonal antibody to yEVenus.

### Table 1. Destination vectors created in this study.

| Plasmid        | Marker | GenBank Acc. No. |
|----------------|--------|-----------------|
| pDEST413TEF17  | HIS3   | JX901379        |
| pDEST414TEF17  | TRP1   | JX901380        |
| pDEST415TEF17  | LEU2   | JX901381        |
| pDEST416TEF17  | URA3   | JX901382        |
| pDEST413CYC17  | HIS3   | JX901383        |
| pDEST414CYC17  | TRP1   | JX901384        |
| pDEST375      | URA3, MET15  | KC614689       |

*15’ and 3’ flanking sequences of MET15.

### Table 2. Entry clones created in this study.

| Plasmid       | Promoter          | ORF               |
|---------------|-------------------|-------------------|
| pYS51         | S. cerevisiae CUP1|                   |
| pYS2         | S. pombe ADH1     |                   |
| pYS3         | S. cerevisiae TEF |                   |
| pYS6        | TetO2-CYC1TATA    |                   |
| pYS7        | TetO2-CYC1TATA    |                   |
| pCG32        | yEGFP            |                   |
| pDHM7        | PEST yEGFP-Cln2   |                   |
| pCG55        | yEVenus          |                   |
| pTS61        | yEVenus-NLS*1     |                   |
| pCG98        | yEVenus-Cln2PEST-NLS |               |
| pCG40        | mCherry          |                   |
| pYS60        | mCherry-NLS      |                   |
| pYS19        | rTA               |                   |
| pYS20        | rTA               |                   |
| pTS58        | AIDrtTA          |                   |
| pYS57        | AIDrtTA          |                   |
| pCG72        | OsTIR1-9Myc      |                   |

Promoter Entry clones were created with pDONR221PS-P2 and ORF Entry clones with pDONR221P1-PSr.

*1Significant cytoplasmic fluorescence was observed when overexpressed, for example, by TEF promoter.

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| pDEST416TEF17  | URA3   | JX901382        |
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anti-TetR antibody (Fig. 5A and B; note that different anti-TetR and anti-Myc antibodies were used in panel A and B). As shown in Fig. 5A, AIDtTA was efficiently degraded by 3 hours following the addition of NAA. This degradation depended on the expression of OsTIR1 (tagged with 9xMyc epitope). The AID was also required for the degradation as the expression of untagged tTA was not affected in YS114 strain in the presence of NAA (Fig. 5B). Likewise, AIDrTA was degraded in NAA-dependent manner in YS114 strain (with OsTIR1), but not in YS129 (without OsTIR1) (Fig. 5C). When AIDrTA was expressed by the strong TEF promoter in YS114 strain, a fraction of AIDrTA protein remained intact even 4 hours after the addition of NAA to the medium (Fig. 5D).

Next, we verified the activity of AIDrTA as a transcriptional activator. YS129 yeast strain was transformed with the reporter plasmid construct pCG87 (TetO7-mCherry-NLS; mCherry fluorescent protein reporter construct) together with pCG84 (ADH1-tTA) or pDHM19 (ADH1-AIDrTA). As shown in Fig. 6, tTA and AIDrTA are active in the absence of doxycycline (−DOX). Because tTA or AIDrTA activity is supposed to be suppressed in the presence of DOX, mCherry reporter fluorescence should be reduced after treating cells with DOX. After 7 hours following the addition of DOX to the medium, however, significant fluorescence still remained (Fig. 6B and C; +DOX). This is presumably because the half-life of mCherry is relatively long and it may take longer to see the reduction of the fluorescence after addition of DOX, which is consistent with the results of flow cytometry (see below).

We asked whether any rapid reduction of fluorescence after the suppression of AIDrTA activity by DOX and NAA can be achieved using yEVenus-Cln2PEST as a reporter. For this, we used a diploid strain (JW003) with a copy of ADH1-OsTIR1 and a reporter TetO7-yEVenus-Cln2PEST genes integrated in the genome. JW003 was transformed with pDHM19 (ADH1-AIDrTA). Note that both AIDrTA and OsTIR1 was expressed by constitutively active ADH1 promoter. The yeast cells were then cultured in the presence or absence of NAA or DOX alone, or both together. Fig. 7A shows a schematic diagram of the gene regulation by AIDrTA in this strain. Without the inducers DOX or NAA, Venus (YFP) fluorescence was clearly visible in the nucleus (Fig. 7B). Cells without the plasmid (pDHM19; ADH1-AIDrTA) showed no fluorescence (data not shown). After 1 hour of treatment with DOX, NAA or both, no obvious decrease of fluorescence was observed (Fig. 7C, upper left panels). The addition of DOX took effect in 2 hours, with much reduced fluorescence in the presence of the inducer either alone or together with NAA (Fig. 7C, upper right panels). It took 3 hours for NAA alone to suppress effectively the expression of the reporter yEVenus-Cln2PEST (Fig. 7C, lower left panels). By 4 hours after the addition of the inducers, fluorescence was greatly reduced in all cases (Fig. 7C, lower right panels). The cells treated with both DOX and NAA showed more pronounced reduction of fluorescence than those with either DOX or NAA alone. These results indicate that AIDrTA activity can be reduced by DOX and NAA.
controlled efficiently by DOX and NAA. DOX took effect faster than NAA perhaps partly because DOX directly inhibits the reporter gene induction by AIDtTA while NAA acts indirectly by promoting the degradation of AIDtTA. We tested two concentrations of NAA at 0.5 mM and 2 mM; increasing the concentration of NAA did not accelerate the reduction of the fluorescence (the results with 2 mM NAA is shown in Fig. 7). We also performed a similar experiment using YS114 strain (with an

**Table 3. Expression plasmid vectors constructed by Gateway recombination method in this study.**

| Plasmid | Promoter | ORF | Marker | Destination vector |
|---------|----------|-----|--------|--------------------|
| pCG52   | S. cerevisiae TEF | mCherry | LEU2  | pDEST415TEFt7 |
| pCG109  | S. pombe ADH1 | mCherry-NLS | TRP1 | pDEST414TEFt7 |
| pCG57   | S. cerevisiae TEF | yEVenus | LEU2  | pDEST415TEFt7 |
| pRN1    | S. pombe ADH1 | yEVenus-NLS | LEU2  | pDEST415TEFt7 |
| pDHM57  | S. pombe ADH1 | yEVenus-Cln2PEST-NLS | LEU2  | pDEST415TEFt7 |
| pCM25   | S. cerevisiae CUP1 | yEVenus-Cln2PEST-NLS | LEU2  | pDEST415TEFt7 |
| pCG87   | TetO-CYC1TATA | mCherry-NLS | TRP1 | pDEST414TEFt7 |
| pCG103  | TetO-CYC1TATA | yEVenus-Cln2PEST-NLS | TRP1  | pDEST414TEFt7 |
| pCM20   | TetO-CYC1TATA | yEVenus-Cln2PEST-NLS | TRP1  | pDEST414TEFt7 |
| pCG84   | S. pombe ADH1 | tTA  | HIS3  | pDEST413TEFt7 |
| pCG85   | S. pombe ADH1 | rtTA | HIS3  | pDEST413TEFt7 |
| pDHM19  | S. pombe ADH1 | AIDtTA | HIS3  | pDEST413TEFt7 |
| pDHM20  | S. pombe ADH1 | AIDtTA | HIS3  | pDEST413TEFt7 |
| pCG112*1 | S. cerevisiae TEF | tTA  | HIS3  | pDEST413TEFt7 |
| pCG113  | S. cerevisiae TEF | rTA  | HIS3  | pDEST413TEFt7 |
| pCG106*1 | S. cerevisiae TEF | AIDtTA | HIS3  | pDEST413TEFt7 |
| pCG107  | S. cerevisiae TEF | AIDtTA | HIS3  | pDEST413TEFt7 |
| pMM*6   | S. cerevisiae TEF | AIDtTA | HIS3  | pDEST413TEFt7 |
| pCG81   | S. pombe ADH1 | OsTIR1-9Myc | URA3 | pDEST416TEFt7 |

*1Yeast cells with these expression vectors showed poor growth with an unknown reason.

*2Integration vector.

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integrated \textit{ADH1-OsTIR1} transformed with two plasmids \textit{pDHM19} (\textit{ADH1-AIDtTA}) and \textit{pCG103} (\textit{TetO} -\textit{yEVenus-Cln2PEST}) had similar profile of reduction in fluorescence observed after cells were treated with DOX and NAA, although the level of fluorescence was more heterogeneous across the cell population in all conditions tested than that in JW003 strain (data not shown).

The same set of yeast strains used in Fig. 6, i.e., YS129 or YS114 with two vectors \textit{pCG87} (mCherry reporter plasmid) and \textit{pCG84 (ADH1-tTA)} or \textit{pDHM19 (ADH1-AIDtTA)} were subjected to flow cytometry to examine the gene expression control by AIDtTA (Fig. 8A–C). Cells were treated with either doxycycline (+DOX; broken green lines) or NAA alone (+NAA; cyan), or both (+DOX & NAA; orange) for 7 hours, or left untreated (no inducers; broken magenta). Consistent with the observation by fluorescence microscopy (Fig. 6), tTA and AIDtTA were equally active in the absence of doxycycline and NAA, although the level of fluorescence was more heterogeneous across the cell population in all conditions tested than that in JW003 strain (data not shown).

In the presence of NAA (cyan lines), the mCherry expression decreased only in the cells with both AIDtTA and OsTIR1 expressed (Fig. 8C). These results are consistent with the \textit{NAA-} and \textit{OsTIR1-dependent} degradation of AIDtTA detected by Western blots. We also tested the doxycycline-dependent gene expression by AIDrtTA and its degradation in OsTIR1- and NAA-dependent manner (Fig. 9). The YS114 (with OsTIR1) and YS129 (without OsTIR1) strains transformed with the plasmids \textit{pCG87 (mCherry reporter construct)} and \textit{pCG107 (TEF-AIDrtTA)} (Fig. 9A) were cultured in the presence or absence of DOX or NAA for 5 hours, and observed by fluorescence microscopy (Fig. 9B). Expression of the fluorescent reporter mCherry was detected in response to +DOX in both YS114 and YS129 strains (Fig. 9B, middle panels). In contrast, when DOX was added to the cell together with NAA (+DOX&NAA), the reporter expression was inhibited in YS114 strain (Fig. 9B, right bottom panels). The expression of mCherry was not affected in the absence of OsTIR1 after addition of NAA (Fig. 9B, left bottom panels). DOX-dependent expression of reporter fluorescent proteins was also observed using the strain with an integrated copy of TEF-AIDrtTA (MM017; Fig. 9C).

Those yeast strains used for fluorescence microscopy were also examined by flow cytometry (Fig. 8D–F). The results confirmed the DOX-dependent activation (green broken line) by AIDrtTA (Fig. 8E and F). The data also demonstrated the NAA-dependent reduction (orange line) of fluorescent reporter proteins in cells expressing AIDrtTA and OsTIR1 (Fig. 8F). In Fig. 8D–F, the data for cells treated with NAA alone are not shown, which have similar distribution of fluorescence as those of untreated ones (no inducers; broken magenta lines). YS129 (the strain without OsTIR1) with \textit{TEF-AIDrtTA} showed around five fold decrease in fluorescence when DOX was added together with NAA (Fig. 8E, green broken and orange lines); the reason for this reduction in...
fluorescence remains unknown. We also found that when rtTA expression was induced by the strong promoter TEF, it activated the reporter gene in the absence of DOX (Fig. 8D, magenta broken line). Such spurious DOX-independent activation of the TetO promoter by rtTA was not observed when its expression was induced by moderate ADH1 promoter (data not shown).

Discussion

In this study, we have created a set of versatile Gateway Destination vectors and a collection of promoter and ORF Entry clones to facilitate gene network construction. The multisite Gateway technology enables the efficient assembly of a promoter and an ORF in a one-step recombination reaction in vitro. An expression vector generated by this method can readily be introduced into yeast for functional assays of the assembled gene. A similar combinatorial approach was demonstrated to be an efficient method for producing diverse phenotypes of synthetic networks in E. coli [14]. The Destination vector pDEST375 described above allows a stable single-copy integration of an artificial gene at MET15 locus, adding a flexible option for engineering artificial gene networks in yeast. We plan to construct similar Gateway vectors for integration at the other chromosome locus utilized by Sadowski et al. [7], such as ADE8, FCY1 and LYS2.

The collection of Entry clones for our gene assembly strategy may be expanded to include a number of other biological parts which has already been characterized and available for synthetic biology applications. For instance, DNA fragments encoding a subset of BioBrick standard biological parts (http://partsregistry.org/) [15] could be converted into Entry clones by PCR amplification using the prefix and suffix sequences that flank every BioBrick part, followed by Gateway BP recombination reactions. Although the Entry clones presented here are for two-fragment recombinations, the Destination vectors can be used for recombination reactions of three or more DNA fragments. This would be useful for the assembly of a gene with a complex promoter.

In an effort to increase the flexibility of synthetic gene networks in yeast, we have also constructed two new tunable transcriptional transactivators, AIDtTA and AIDrtTA. We showed that when O. sativa TIR1 gene is co-expressed, they were rapidly degraded in response to auxin. We also demonstrated that the expression of the reporter gene by AIDtTA and AIDrtTA were controlled efficiently by NAA and/or DOX added to the medium (Fig. 6–9). We constructed a destabilized version of Venus, yEVenus-Cln2PEST, and used it to monitor the AIDtTA’s activity (Fig. 7). Because Venus has faster, improved maturation properties of the chromophore [16], yEVenus-Cln2PEST may be better-suited than the original version, GFP-Cln2PEST [10], as a reporter of the dynamics of synthetic gene networks. A unique advantage of AIDtTA or AIDrtTA may be that the ‘memory’ of gene activation can be erased rapidly by an auxin-regulated protein degradation. The rapid degradation of these transactivators are however dependent on the fine balance of their expression level and that of Tir1 protein: when AIDrtTA was expressed by the strong TEF

Figure 5. Auxin-induced degradation of AIDtTA and AIDrtTA detected by Western blotting. OsTIR1 is tagged with 9xMyc epitope. Cells were treated with NAA or left untreated for the periods as indicated. (A) The yeast strain with an integrated copy of OsTIR1 gene (::ADH1-OsTIR1; YS114 strain) and ADH1-AIDtTA plasmid. (B) Control strain YS129 with ADH1-AIDtTA, YS114 with ADH1-tTA, and YS114 with ADH1-AIDtTA plasmid. (C) YS129 and YS114 with ADH1-AIDrtTA plasmid. (D) YS129 with TEF-AIDrtTA, YS114 with TEF-tTA and YS114 with TEF-AIDrtTA plasmid. Cells were harvested and subjected to Western blotting using anti-TetR (α-TetR, for tTA, AIDtTA, rtTA and AIDrtTA) and anti-Myc (α-Myc, for OsTIR1) antibodies. Note that different primary antibodies were used in Fig. 5A (indicated by *1 and *2) and B–D (*3 and *4); see Materials and Methods. Arrowheads indicate non-specific proteins recognized by the primary antibodies, which act as loading controls.

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promoter, its degradation was retarded (Fig. 5D). The AID system therefore requires fine-tuning of the expression level of its components to achieve the desired functionality. For example, the expression level of OsTIR1 in yeast may be increased by adopting a codon-optimized version of the ORF [17], thereby improving the efficiency of the degradation of AID-tagged target proteins.

An additional control may also be integrated into synthetic networks using these engineered transactivators by making the TIR1 gene expression inducible. AIDtTA and AIDrtTA may be combined together with other biological logic components to achieve the desired functionality. For example, AIDtTA and N-IMPLY, using RNA binding proteins that inhibit the translation of transcripts harboring specific RNA motifs [18]. AIDtTA and AIDrtTA may be combined together with other biological logic operation devices in eukaryotes [18–21].

The budding yeast Saccharomyces cerevisiae is an attractive system for synthetic biology because of its vast resources and tools for biotechnology applications. Moreover, S. cerevisiae has been explored as a platform for synthesizing a complete genome [22,23] and chromosome arms [24]. These studies have presented a prospect of engineering a large-scale artificial gene network in yeast. Although the number of biological parts customized for yeast synthetic biology is still limited, it is rapidly growing in recent years [25–27]. The multisite Gateway recombination method for assembling artificial genes may be a useful addition to the toolbox for synthetic biology in yeast and also a valid approach in other organisms.

Materials and Methods

Yeast Manipulations, Strains and Media

Genetic manipulations and transformations of yeast cells were performed as described [28]. After transformation, cells were plated on synthetic complete medium (SC) lacking appropriate amino acids [28]. Transformed cells with plasmids were cultured in the selective media until the culture reaches early to mid-log phase of growth ($OD_{600} = 0.1 \sim 1.0$) for experiments. The yeast strains used in this study are FY1679 (MATa/gAL2/ GAL2HIS3/ his3A200LEU2 / leu2A1TRP1 / trp1A63ura3 – 52 / ura3 – 52; EUROSCARF Acc. No. 10000D), YS114 (MATz/gAL2/ his3A200 trp1A63ura3 – 52; ADH1 – OsTIR1 – Myc; URA3; this study), MM017 (MATa/ his3A200 leu2A0trp1A63ura3A0 met15 :: TEF – AIDtTA; this study), YS008 (MATz/ his3A1leu2A0trp1A63ura3A0 met15 :: ADH1 – OsTIR1 – Myc; this study), YS017 (MATa/ his3A1leu2A0trp1A63ura3A0 met15 :: TetO7 – yEVenus – Cln2Pest – NLS; this study), YS081 (MATa/ his3A1leu2A0trp1A63ura3A0; this study), JW003 (MATa/ his3A1/ his3A1 leu2A0trp1A63ura3A0 trp1A63ura3A0 met15 :: ADH1 – OsTIR1 – Myc/ MET15LYS2/ lys2 :: TetO7 – CYC1TATA – yEVenus – Cln

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Figure 6. Fluorescent reporter protein expression induced by AIDtTA. YS129 strain was transformed with the plasmid constructs indicated above each panel. The activity of tTA and AIDtTA was detected by mCherry fluorescent reporter protein expression. (A) TetO7 - mCherry-NLS (reporter plasmid) only, without doxycycline (-DOX). (B) ADH1-tTA and TetO7-mCherry-NLS. (C) ADH1-AIDtTA and TetO7-mCherry-NLS. Cells were observed 7 hr after addition of DOX. DIC: differential interference contrast; mCherry: mCherry fluorescence was detected using TRITC filter. Scalebar = 5 μm. doi:10.1371/journal.pone.0064419.g006
PEST — NLS; this study) and YS129 (MATahis3 Δleu2Δ0trp1Δ63ura3Δ0; this study), which are all congenic with S288C. To integrate the *Oryza sativa* TIR1 gene, pNHK53 (an integration vector of *ADH1-OsTIR1-9Myc* [8]) was linearized by StuI and transformed into FY1679. To derive YS114, the diploid strain with an integrated pNHK53 was sporulated. Sporulations were performed as follows: Freshly streaked cells on YPD plate were patch-streaked on GNA (5% glucose, 3% Difco nutrient broth (BD Bioscience; cat. no. 234000), 1% yeast extract, 2% bacto-agar) and incubated overnight at 30°C. The cells on GNA were patch-streaked again on GNA and incubated overnight at 30°C. A small quantity of cells (equivalent to a medium-sized colony on a plate) was then suspended in 2.5 ml of sporulation medium (1% potassium acetate, 0.5% zinc acetate) and incubated at 25°C with vigorous shaking for up to a week. The genetic selection of YS008, YS017 and MM017 strains were performed according to Sadowski *et al.* [7], which describes the detailed methods of gene integration and disintegration. To create MM017, pMM6 (Table 3) was linearized with MluNI (an isoschizomer of MscI) and transformed into YS129 for integration. To derive YS008, pCM33 (integration vector of *ADH1-OsTIR1-9Myc*; see below for its construction) was linearized by MluNI and transformed into YS129 for integration. To make YS017, pCM36 (*TetO*7-*CYC1TATA-yEVenus-Cln2PEST-NLS*; see below for its construction) was linearized by NruI and transformed into YS081 strain. JW003 strain was made by mating YS008 and

![Figure 7: Suppression of AIDtTA activity by doxycycline and NAA.](https://example.com/figure7)

The yeast strain with an integrated copy of *ADH1-OsTIR1* and a reporter *TetO*7-*yEVenusCln2PEST-NLS* gene was transformed with the plasmid harboring AID1-AIDtTA. The fluorescent reporter protein expression was examined in the presence or absence of DOX and NAA as indicated. (A) Schematic diagram of the gene network of the strain. (B) Expression of yEVenusCln2PEST in the absence of DOX and NAA. (C) Expression of yEVenusCln2PEST after treatment with DOX or NAA alone or both for 1, 2, 3 and 4 hours. DIC: differential interference contrast; YFP: YFP filter channel. Scalebar = 5 μm.

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![Figure 8: Gene expression control by AIDtTA and AIDrtTA: flow cytometry analysis.](https://example.com/figure8)

(A–F) The yeast strains YS129 (-OsTIR1) and YS114 (+OsTIR1) were transformed with a reporter plasmid construct (*TetO*7-*mCherry-NLS*) together with the plasmid harboring the gene as labeled on each panels. These transformed yeast cells were treated with either doxycycline (+DOX; broken green lines) or NAA alone (+NAA; cyan), or both (+DOX & NAA; orange) for 7 hours, or left untreated (no inducers; broken magenta), and analyzed by flow cytometry. Fluorescence of 50,000 cells for each sample were measured. The data were gated to remove those of cell debris and aggregates, binned in log scale (0 to 10^5, 100 bins) and plotted. The plots are offset at fluorescence = 4 arbitrary units.

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YS017. JW003 transformed with the plasmid pDHM19 (ADH1-AIDtTA) was used for the experiment shown in Fig. 7.

To test the regulation of AIDtTA and AIDrtTA by doxycycline and auxin (Fig. 5 to 9), YS114, MM017 and YS129 strains were transformed with pCG103 (TetO7-CYC1TATA-yEVenus-Cln2-PREST-NLS), pCG87 (TetO7-CYC1TATA-mCherry-NLS), pCG84 (ADH1-tTA), pDHM19 (ADH1-AIDtTA), pCG85 (ADH1-rtTA), pDHM20 (ADH1-AIDrtTA), pCG113 (TEF-rtTA), and pCG107 (TEF-AIDrtTA) as indicated in each figure. Doxycycline (DOX) was added to cell cultures at the final concentration of 5 mg/ml. For the induction of protein degradation using AID system, 1-Naphthaleneacetic acid (NAA; potassium salt, Sigma cat. no. N1145) was added to cell cultures to 0.5 mM, except the experiment shown in Fig. 7 in which 2 mM was used. We observed little inhibition of cell growth up to 2 mM of NAA. Phosphate-citrate buffer (64.2 mM Na2HPO4 and 17.9 mM citric acid, pH 6.0) was added to SC medium to prevent the acidification of the culture [29]. To induce CUP1 promoter, copper nitrate (Cu(NO3)2, Sigma cat. no. 223395) was added to the medium to 0.25 mM. DOX, NAA, or copper nitrate were added to cell cultures at OD600 = 0.2.

Molecular Biology Techniques

Standard molecular biology techniques were used for DNA manipulations. DNA fragments were purified using a PCR purification kit or gel purification kit (Qiagen). DNA ligation was performed using a rapid DNA ligation kit (Roche Applied Science). For PCR reactions, Phu polymerase (Promega) or Velocity DNA polymerase (Bioline) were used. PCR primers used in this study are listed in Table 4. Plasmid clones created with PCR reactions were verified by DNA sequencing. The Gateway recombination reactions were performed using Multisite Gateway Pro Kits (Life Technologies). The donor vectors (pDONR221P1-P5, pDONR221P5-P2; Life Technologies), and destination vectors were prepared using ccdB Survival T1R Chemically Competent E. coli (Life Technologies).

Construction of destination vectors. The destination vectors containing a yeast centromere were built upon the pRS vector series [pRS413, pRS414, pRS415 and pRS416] [6]. A. gossypii TEF terminator was amplified by PCR from pAG25 [30], using the standard T7 sequencing primer and TEF-F primer. The amplified DNA was cut with NotI and SacI and cloned into the pRS vectors to create pRS413TEF1, pRS414TEF1, pRS415TEF1 and pRS416TEF1. S. cerevisiae CYC1 terminator was amplified from pCM183 [31] by PCR with CYC1F and CYC1R primers, cut

Figure 9. Gene expression control by AIDrtTA. (A) Schematic representation of the experimental system. AIDrtTA activity was detected by mCherry fluorescent reporter protein expression. (B) Yeast strains as indicated were cultured in the presence of doxycycline (+DOX), auxin (+NAA), both doxycycline and auxin (+DOX & NAA), or in the absence (no inducer). Cells were observed 6 hrs after addition of the inducers. (C) Yeast strain with an integrated copy of TEF-AIDrtTA (MM017) was cultured in the presence or absence of doxycycline for 5 hours. DIC: differential interference contrast; mCherry: mCherry fluorescence detected with TRITC filter. Scalebar = 5 μm.

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with NotI and SacI and cloned into the pRS413 vector digested with the same enzymes to create pRS413CYC1T. A cassette containing two recombination sites, attR1 and attR2, flanking the ccdB and CmR (chloramphenicol resistance) genes was amplified by PCR from pDEST17 (Invitrogen), using primers attR1-F and attR2-R. The PCR product was digested by SpeI and AvrII and cloned into pRS413CYC1T to yield pDEST413CYC1T. The attR1-attR2 cassette with the ccdB and CmR genes was cut out from pDEST413CYC1T by XhoI and XbaI and cloned into pRS413TEFt, pRS415TEFt and pRS416TEFt, which yielded pDEST413TEFt, pDEST415TEFt and pDEST416TEFt, respectively. To create pDEST414TEFt, 2 kb DNA fragment containing the cassette and the TEF terminator was cut out from pDEST413TEFt by XhoI and SacI and ligated to pRS414 digested with the same enzymes. Similarly, the XhoI-SacI DNA fragment from pDEST413TEFt was cut out from pDEST414TEFt and cloned to pRS414 digested with the same enzymes. Although these pDEST plasmids were functional as destination vectors, T7 terminator downstream of ccdB gene was found to be required for efficient selection of expression clones. Therefore, DNA fragments harbouring the T7 terminator were cut out from pDEST17 and cloned into these vectors, to create pDEST413TEFt7, pDEST414TEFt7, pDEST415TEFt7, pDEST416TEFt7, pDEST413CYC1t7 and pDEST414CYC1t7 (Fig.1). pDEST375 was made by isolating the KpnI/SacI DNA fragment containing the Gateway attR1-attR2 cassette from pDEST416TEFt7 and cloning it into pIS375 [7] using the same restriction sites.

**Construction of promoter entry clones.** *S. cerevisiae CUP1* promoter was amplified by PCR from pYM-N1 [11] using the oligonucleotide primers B2-CUP1-F and B3-CUP1-R. *S. pombe ADH1* promoter was amplified from pCM245 [32] using B2-ADH-F and B3-ADH-R primers. *S. cerevisiae TEF* promoter was amplified from pYM-N18 [11] using the primers B2-TET-F and

| Primer          | Sequence                     |
|-----------------|------------------------------|
| T7              | TAATACGACTCTACATATAGG        |
| TEFt-F          | TTGCGGCCGCTCAGTACTGACAATAA   |
| CYC1F           | AAGCGGCCGCATCAGTAACTAGT      |
| CYC1R           | TTGAGCTCTAAATAGAAGCGCTTCCAGG |
| attR1-F         | ACCACTAGTACAAGTTTGTACAAAAA   |
| attR2-R         | GTTCCTAGGACCACTTTGTACAAGAA   |
| B2-CUP1-F       | GGGGACCACCTTTGTAAGAGGATAGTC  |
| B2-ADH-F        | GGGGACCACCTTTGTAAGAGGATTC    |
| B2-TET-F        | GGGGACCACCTTTGTAAGAGGATGT    |
| B2TetO-1        | GGGGACCACCTTTGTAAGAGGATAGTC  |
| B5tetO-1        | GGGGACCACCTTTGTAAGAGGATAGTC  |
| B5r-tTA         | GGGGACAACTTTTGAATGAGGATAGTC  |
| B1-tTA          | GGGGACAACTTTTGAATGAGGATAGTC  |
| AIDtTA-F        | GTTGAGACGGCTGAGGTTGCTGAC    |
| AIDtTA-R        | TTATTTTTATTCCTACATGAGGCT    |
| B5r-AIDGFPNLS   | GGGGACAACTTTTGAACATTGAGGATAGTC |
| B5r-yEGFP-F2    | GGGGACAACTTTTGAATGAGGATAGTC |
| B1-yEGFP-R2     | GGGGACAACTTTTGAACATTGAGGATAGTC |
| B1-yVenus-R2    | GGGGACAACTTTTGAACATTGAGGATAGTC |
| B1-Cln2PESL     | GGGGACAACTTTTGAACATTGAGGATAGTC |
| B1-yEGFPNL5-R   | GGGGACAACTTTTGAACATTGAGGATAGTC |
| B5-mCherry-F1   | GGGGACAACTTTTGAACATTGAGGATAGTC |
| B1-mCherry-R1   | GGGGACAACTTTTGAACATTGAGGATAGTC |
| B1-mCherryNL5-R | GGGGACAACTTTTGAACATTGAGGATAGTC |
| XmAPEST         | ATACCCCGGGCCTGCTTGCTGTCATCCAG |
| XmAPESTR        | CTACCCGAGCATCTGCTGGTTCATCCAG |
| B5r-TIR1        | GGGGACAACTTTTGAACATTGAGGATAGTC |
| B1-TIR1         | GGGGACAACTTTTGAACATTGAGGATAGTC |

**Table 4. PCR primers.**

**Primer** | **Sequence** |
|-----------|--------------|
| T7        | TAATACGACTCTACATATAGG |
| TEFt-F    | TTGCGGCCGCTCAGTACTGACAATAA |
| CYC1F     | AAGCGGCCGCATCAGTAACTAGT |
| CYC1R     | TTGAGCTCTAAATAGAAGCGCTTCCAGG |
| attR1-F   | ACCACTAGTACAAGTTTGTACAAAAA |
| attR2-R   | GTTCCTAGGACCACTTTGTACAAGAA |
| B2-CUP1-F | GGGGACCACCTTTGTAAGAGGATAGTC |
| B2-ADH-F  | GGGGACCACCTTTGTAAGAGGATTC |
| B2-TET-F  | GGGGACCACCTTTGTAAGAGGATGT |
| B2TetO-1  | GGGGACCACCTTTGTAAGAGGATAGTC |
| B5tetO-1  | GGGGACCACCTTTGTAAGAGGATAGTC |
| B5r-tTA   | GGGGACAACTTTTGAATGAGGATAGTC |
| B1-tTA    | GGGGACAACTTTTGAATGAGGATAGTC |
| AIDtTA-F  | GTTGAGACGGCTGAGGTTGCTGAC |
| AIDtTA-R  | TTATTTTTATTCCTACATGAGGCT |
| B5r-AIDGFPNLS | GGGGACAACTTTTGAACATTGAGGATAGTC |
| B5r-yEGFP-F2 | GGGGACAACTTTTGAATGAGGATAGTC |
| B1-yEGFP-R2 | GGGGACAACTTTTGAATGAGGATAGTC |
| B1-yVenus-R2 | GGGGACAACTTTTGAATGAGGATAGTC |
| B1-Cln2PESL | GGGGACAACTTTTGAATGAGGATAGTC |
| B1-yEGFPNL5-R | GGGGACAACTTTTGAATGAGGATAGTC |
| B5-mCherry-F1 | GGGGACAACTTTTGAATGAGGATAGTC |
| B1-mCherry-R1 | GGGGACAACTTTTGAATGAGGATAGTC |
| B1-mCherryNL5-R | GGGGACAACTTTTGAATGAGGATAGTC |
| XmAPEST    | ATACCCCGGGCCTGCTTGCTGTCATCCAG |
| XmAPESTR   | CTACCCGAGCATCTGCTGGTTCATCCAG |
| B5r-TIR1   | GGGGACAACTTTTGAATGAGGATAGTC |
| B1-TIR1    | GGGGACAACTTTTGAATGAGGATAGTC |

**PCR primers used in this study.** Sequences are in 5’ to 3’ direction.

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B5-TET-R. A DNA fragment containing TetO2 operator with the TATA region of S. cerevisiae CYC1 gene (CYC1TATA) was amplified from pCM171 [31] using the primers B2-TetO-1 and B3-TetO-1. Likewise, TetO2 with CYC1TATA was amplified from pCM159 [31] using the same pair of primers. These PCR products were purified and subjected to BP recombination reaction with pDONR221P5-P2 in vitro to create the plasmids listed in Table 1.

### Construction of ORF entry clones.

yEGFP ORF was amplified from pYM25 [11] using the primers B5r-yEGFP-F2 and B1-yEGFP-R2. yEGFP-Cln2 ORF amplified from pSVA13 [10] using B5r-yEGFP-F2 and B1-Cln2PEST. yEVenus ORF was amplified from pKT90 [33] using the primer pair B5r-yEGFP-F2 and B1-yEVenus-R2. mCherry ORF were amplified from pCS-memb-mCherry (mCherry fused to a glycine linker and a SV40 nuclear localization signal (NLS; amino acid sequence PGGGGPKKKRKVD) was added to the C-terminus of yEVenus ORF. mCherry-F1 and B1-mCherry-R1 primers. A glycine linker and a TATA region of P. pastoris CYC1 (PCR product 2). PCR product 1 and 2 have an overlapping sequence: pCG98 was created by inserting a Cln2PEST Xmal fragment into pYS61. The Cln2PEST sequence was amplified by PCR using primers Xmal-PESTF and Xmal-PESTR, and pSVA13 as a template. tTA and rtTA ORFs were amplified from pCM171 [31] and pCM251 [32] respectively, using the primers B5r-tTA and B1-tTA. To create pCG72, OsTIR1-9Myc was amplified by PCR using primers B5r-TIR1 and B1-TIR1, and pNHK36 [8] as a template. The amplified ORFs were cloned into pDONR221P1-P5r by Gateway BP reaction to create the Entry clones in Table 2. Auxin inducible degron (AID) was fused to tTA and rtTA using a method that combines a Gateway BP reaction in vivo and a homologous recombination in vivo in E. coli [13]. For this, two PCR reactions were performed. First, a DNA fragment encoding AID was amplified from pNHK12 [8] using the oligonucleotides AIDtTA- and B5r-AIDGFPNL5-R (PCR product 1). Second, tTA and rtTA ORF were amplified using the primers AidtTA-F and B1-tTA (PCR product 2). PCR product 1 and 2 have an overlapping sequence that mediates a homologous recombination in vivo. These PCR products together with pDONOR221P1-P5r were subjected to a BP reaction, followed by a transformation into E. coli (DH5α strain), to create pYS57 (Entry clone of AidtTA) and pYS58 (AIDtTA).

### Other plasmids.

The integration vector pCM33 was made as follows: pCG81 (Table 3) was digested by SphI/BapQI and blunt-ended by T4 DNA polymerase (New England Biolabs). The fragment containing ADH1-OsTIR1-9Myc was purified and ligated to pLS75 [7], which was linearized by BamHI and blunt-ended by Klenow fragment (Promega). pCM36 was created by inserting PvuII fragment of pCM20 (Table 3) into pLS895 [7], which was cut by BamHI and blunt-ended by Klenow fragment. pNHK12, pNHK36 and pNHK53 were obtained from the National Bio-Resource Project (NBRP) of MEXT, Japan. The following plasmids were obtained from EUROSCARF (EUROpean Saccharomyces Cerevisiae ARchive for Functional Analysis): pAG25, pCM159, pCM171, pCM183, pCM245, pCM251, pBS375, pKT90, pYMP25, pYM-N1, pYM-N18.

### Microscopy and Flow Cytometry.

Yeast cells were cultured in liquid SC medium lacking appropriate amino acids and mounted on a glass slide for fluorescence microscopy. Images were acquired using a Zeiss Axioplan2 microscope with an oil-immersion objective lens (100×) equipped with a CCD camera (Hamamatsu ORCA-ER) and Openlab software (Perkin Elmer). For flow cytometry, cells were harvested by centrifugation, or by vacuum filtration on nitrocellulose membranes (1.2μm, Millipore, cat. no. RAWP02500), re-suspended in ice-cold 1×PBS and sonicated briefly before subjected to the analysis using LSRFortessa (BD Biosciences). Fluorescence of 50,000 cells for each sample was measured by flow cytometry. Based on the FFS/SSC values, the data were gated to remove cell debris and aggregates. The raw data for the remaining cells, between 86% and 98% of the original sample, were exported to MATLAB (MathWorks, Inc., 2012) and binned in log scale (0to10×100bins). The scale of each bin was normalized by the sum of all cells in the respective sample and plotted (Fig. 8). No smoothing has been applied.

### Western Blotting.

Protein extraction from yeast cells was conducted as described [34]. Standard techniques were used for electrophoresis (SDS-PAGE) and Western blotting. Western blotting was performed using Amersham ECL Plus Western Blotting detection reagent (cat. no. RPN2132). The primary antibodies used were anti-myc monoclonal (1:1000, Millipore, 9E10, *2 in Fig. 5A) or rabbit polyclonal antibody (1:5000, Abcam, cat. no. ab9106, *4 in Fig. 5B–D) and TetR monoclonal antibody (1:1000, Clontech, cat. no. 631108, *1 in Fig. 5A; 1:1000, MoBiTec, TET02, *3 in Fig. 5B–D); the secondary antibodies were anti-mouse IgG HRP-linked antibody (1:1000, Cell Signaling #7076) and anti-rabbit IgG HRP-linked antibody (1:1000, DAKO, cat. no. P0217). Equal loading of protein extracts in SDS-PAGE was verified by Ponceau S staining of blotted membranes.

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### Author Contributions.

Conceived and designed the experiments: YS CVG. Performed the experiments: CVG MM YS. Analyzed the data: CVG MM YS. Wrote the paper: YS CG.

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