

Dual Role of the *Saccharomyces cerevisiae* TEA/ATTS Family Transcription Factor Tec1p in Regulation of Gene Expression and Cellular Development

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In *Saccharomyces cerevisiae*, the transcription factors Tec1p and Ste12p are required for haploid invasive and diploid pseudohyphal growth. Tec1p and Ste12p have been postulated to regulate these developmental processes primarily by cooperative binding to filamentous and invasion-responsive elements (FREs), which are combined enhancer elements that consist of a Tec1p-binding site (TCS) and an Ste12p-binding site (PRE). They are present in the promoter regions of target genes, e.g., *FLO11*. Here, we show that Tec1p efficiently activates target gene expression and cellular development in the absence of Ste12p. We further demonstrate that TCS elements alone are sufficient to mediate Tec1p-driven gene expression by a mechanism termed TCS control that is operative even when Ste12p is absent. Mutational analysis of *TEC1* revealed that TCS control, *FLO11* expression, and haploid invasive growth require the C terminus of Tec1p. In contrast, the Ste12p-dependent FRE control mechanism is sufficiently executed by the N-terminal portion of Tec1p, which contains the TEA/ATTS DNA-binding domain. Our study suggests that regulation of haploid invasive and diploid pseudohyphal growth by Ste12p and Tec1p is not only executed by combinatorial control but involves additional control mechanisms in which Ste12p activates *TEC1* expression via clustered PREs and where Tec1p regulates expression of target genes, e.g., *FLO11*, by TCS control.

Saccharomyces cerevisiae is a dimorphic fungus that interconverts between unicellular and multicellular filamentous growth modes (15). Upon nitrogen starvation, diploid cells switch from growth as single yeast form cells to a filamentous form consisting of chains of elongated cells called pseudohyphae. A related phenomenon called invasive growth is observed in haploid cells (44). In contrast to pseudohyphal growth, haploid invasive growth is not triggered by nitrogen starvation but occurs on rich medium in response to glucose depletion (7). In addition, low concentrations of α -factor mating pheromone induce haploid invasive growth (9, 43).

Both haploid invasive growth and diploid pseudohyphal development require transcription factors Tec1p and Ste12p (12, 27, 31, 34, 44). Tec1p was originally identified as a regulator of expression of *Ty1* transposon insertions (24). Tec1p contains an evolutionarily conserved DNA-binding domain that has been named the TEA (TEF-1, Tec1p, and AbaAp) or ATTS (AbaAp, TEF-1, Tec1p, and Scalloped) motif (5). The TEA/ATTS domain is shared by a group of eukaryotic transcription factors, including Tec1p; human TEF-1, which regulates simian virus 40 and papillomavirus type 16 gene expression; and *Aspergillus nidulans* AbaAp, a development-specific transcription factor required for asexual spore formation (2, 5, 52). These transcription factors recognize and bind the conserved sequences CATTCC and CATTCT, which have been termed TEA/ATTS consensus sequences or Tec1p-binding site (TCS)

elements (1, 3, 20, 31). Ste12p was initially identified as a regulator of mating factor-responsive genes that contain pheromone response elements (PREs) matching the consensus sequence TGAAACA in their promoter regions (11, 23, 51). Ste12p binds poorly to a single PRE but binds cooperatively to multiple PREs, leading to efficient transcriptional activation (8, 53). Clustering of PRE elements is common to mating factor-responsive genes (e.g., *FUS1* and *SST2*) and confers not only mating factor-regulated but also cell cycle-regulated transcription (38, 39, 41). Single PREs confer transcriptional activation only if juxtaposed to recognition sites for other transcription factors, e.g., Mcm1p or Tec1p, that bind cooperatively with Ste12p to combined enhancer elements (3, 10, 31).

Combination of one PRE and one TCS element creates an enhancer element that has been termed a filamentation and invasion response element or FRE (31). Ste12p and Tec1p have been demonstrated to bind cooperatively to FREs and to activate gene expression in a synergistic manner (31). FREs are present in the promoter regions of genes involved in haploid invasive growth and diploid pseudohyphal development and include *FLO11*, encoding a cell surface flocculin (29, 46), and *TEC1* itself (31). These findings have led to the view that FRE-mediated gene expression is the major control mechanism by which Tec1p regulates haploid invasion and diploid pseudohyphal growth and that it confers specificity on the distinct Ste12p-regulated developmental programs (31). However, several lines of evidence indicate that FRE control might not be the sole mechanism by which Ste12p and Tec1p control expression of genes required for haploid invasive and pseudohyphal growth. First, the *TEC1* promoter contains several clustered PREs that mediate control of *TEC1* transcription by

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TABLE 1. Strains used in this study

Strain	Relevant genotype ^b	Reference
RH2754	<i>MATa ura3-52 leu2::hisG his3::hisG trp1::hisG</i>	This study
RH2500	<i>MATa tec1Δ::HIS3 ura3-52 leu2::hisG his3::hisG trp1::hisG</i>	This study
RH2755	<i>MATa ste12Δ::TRP1 ura3-52 leu2::hisG his3::hisG trp1::hisG</i>	This study
RH2501	<i>MATa tec1Δ::HIS3 ste12Δ::TRP1 ura3-52 leu2::hisG his3::hisG trp1::hisG</i>	This study
RH2756	<i>MATα tec1Δ::HIS3 ura3-52 leu2::hisG his3::hisG trp1::hisG</i>	This study
RH2757	<i>MATα tec1Δ::HIS3 ura3-52 leu2::hisG his3::hisG</i>	This study
RH2778	<i>MATα tec1Δ::HIS3 ste12Δ::TRP1 ura3-52 leu2::hisG his3::hisG trp1::hisG</i>	This study
RH2758	<i>MATa/MATα tec1Δ::HIS3/tec1Δ::HIS3 ura3-52/ura3-52 leu2::hisG/leu2::hisG his3::hisG/his3::hisG trp1Δ::hisG/TRP1</i>	This study
RH2759	<i>MATa/MATα tec1Δ::HIS3/tec1Δ::HIS3 ste12Δ::TRP1/ste12Δ::TRP1 ura3-52/ura3-52 leu2::hisG/leu2::hisG his3::hisG/his3::hisG trp1Δ::hisG/trp1Δ::hisG</i>	This study
RH2499	<i>MATα tec1Δ::HIS3 FRE(Ty1)-lacZ::LEU2 ura3-52 leu2::hisG his3::hisG trp1::hisG</i>	36
RH2767 ^a	Same as RH2500 except <i>CYC1(ΔUAS)-lacZ::URA3</i>	This study
RH2765 ^a	Same as RH2500 except <i>TCS^{fw}-CYC1-lacZ::URA3</i>	This study
RH2766 ^a	Same as RH2500 except <i>TCS^{bw}-CYC1-lacZ::URA3</i>	This study
RH2764 ^a	Same as RH2500 except <i>TCS^{fw}-6-TCS^{bw}-CYC1-lacZ::URA3</i>	This study
RH2763 ^a	Same as RH2500 except <i>TCS^{fw}-11-TCS^{bw}-CYC1-lacZ::URA3</i>	This study
RH2760 ^a	Same as RH2500 except <i>TCS^{fw}-14-TCS^{bw}-CYC1-lacZ::URA3</i>	This study
RH2761 ^a	Same as RH2500 except <i>TCS^{bw}-13-TCS^{bw}-CYC1-lacZ::URA3</i>	This study
RH2762 ^a	Same as RH2500 except <i>TCS^{bw}-13-TCS^{bw}-13-TCS^{bw}-CYC1-lacZ::URA3</i>	This study
RH2768 ^a	Same as RH2500 except <i>TCS^{fw}-14-PRE^{fw}-CYC1-lacZ::URA3</i>	This study
RH2776 ^a	Same as RH2501 except <i>CYC1(ΔUAS)-lacZ::URA3</i>	This study
RH2774 ^a	Same as RH2501 except <i>TCS^{fw}-CYC1-lacZ::URA3</i>	This study
RH2775 ^a	Same as RH2501 except <i>TCS^{bw}-CYC1-lacZ::URA3</i>	This study
RH2773 ^a	Same as RH2501 except <i>TCS^{fw}-6-TCS^{bw}-CYC1-lacZ::URA3</i>	This study
RH2772 ^a	Same as RH2501 except <i>TCS^{fw}-11-TCS^{bw}-CYC1-lacZ::URA3</i>	This study
RH2769 ^a	Same as RH2501 except <i>TCS^{fw}-14-TCS^{bw}-CYC1-lacZ::URA3</i>	This study
RH2770 ^a	Same as RH2501 except <i>TCS^{bw}-13-TCS^{bw}-CYC1-lacZ::URA3</i>	This study
RH2771 ^a	Same as RH2501 except <i>TCS^{bw}-13-TCS^{bw}-13-TCS^{bw}-CYC1-lacZ::URA3</i>	This study
RH2777 ^a	Same as RH2501 except <i>TCS^{fw}-14-PRE^{fw}-CYC1-lacZ::URA3</i>	This study

^a Strains were obtained by targeted integration of corresponding reporter plasmids (see Table 2) into RH2500 or RH2501.

^b *TCS^{fw}* indicates forward orientation of TCS element, and *TCS^{bw}* indicates backward orientation with respect to the orientation of the TCS element in *FRE(Ty1)* (31).

mating pheromone, by Ste12p and other elements of the mating factor signal transduction pathway, and by the cell cycle (38). Second, microarray analysis has identified several genes that are under the control of Ste12p and Tec1p but that do not contain FRE enhancer elements in their promoter regions, e.g., *PGU1* encoding a secreted endopolygalacturonase that degrades the plant-specific polysaccharide pectin (32). Instead, the promoter regions of these genes often contain several TCS elements that are not neighbored by PRE sites. Third, the TEA/ATTS family transcription factor AbaAp from *A. nidulans* activates gene expression by binding on its own to either single or multiple TCS elements present in the promoter regions of target genes (1). Finally, ectopic expression of the Tec1p-related transcription factors AbaAp from *A. nidulans* and CaTec1p from the human pathogen *Candida albicans* induce *S. cerevisiae* haploid invasive and pseudohyphal growth in strains that lack *STE12* (12, 47).

These findings prompted us to examine the possibility that Ste12p and Tec1p control gene expression and cellular development by mechanisms distinct from FRE-mediated combinatorial control. We provide evidence that *TEC1* gene expression requires Ste12p but is largely independent of Tec1p autoregulation. As a consequence, Tec1p levels drop 20-fold in strains lacking Ste12p. When restored to high levels, Tec1p activates haploid invasive growth and expression of the *FLO11* and *PGU1* genes even in the absence of *STE12*. Tec1p efficiently activates gene expression mediated by synthetic single or combined TCS elements inserted upstream of an upstream activating sequence (UAS)-less reporter gene. This Tec1p-mediated

transcriptional control mechanism, termed TCS control, is operative even when Ste12p is absent. Mutational analysis of *TEC1* reveals that the C terminus of Tec1p is required for haploid invasive growth and TCS-mediated gene expression but is dispensable for the FRE control mechanism. On the basis of our results, we propose that Ste12p and Tec1p control gene expression and cellular development by several distinct mechanisms.

MATERIALS AND METHODS

Yeast strains and growth conditions. All of the yeast strains used in this study are congeneric to the Σ 1278b genetic background (Table 1). The *tec1Δ::HIS3* and *ste12Δ::TRP1* deletion mutations were introduced by using deletion plasmids *ptec1Δ::HIS3* (36) and *pste12Δ::TRP1*. RH2757 was derived from RH2756 by transformation with a linear fragment containing the *TRP1* gene. RH2758 was obtained by mating of RH2500 with RH2757, and RH2759 resulted from mating of RH2501 with RH2778. Standard methods for genetic crosses and transformation were used, and standard yeast culture medium was prepared essentially as previously described (18). When required, synthetic complete (SC) medium lacking appropriate supplements was used. Invasive growth tests were performed as described previously (44). Pseudohyphal development was induced by growth on synthetic low-ammonium medium (15).

Plasmid constructions. All of the plasmids used in this study are listed in Table 2. Plasmids pME2044, pME2045, and pME2047 were constructed by subcloning of a 3.1-kb *PstI-HindIII* fragment carrying *TEC1* from B3366 (34) into YCplac33, YCplac11, and YEplac181, respectively. For regulated expression of *TEC1* from the inducible *GALI-10* promoter, a 2.5-kb *BspI201/SacI* fragment containing a *GALI(p)::TEC1* cassette was subcloned from pME2071 (36) into pRS315, yielding plasmid pME2049. A *SalI* site was inserted after the ATG start codon of *TEC1* by a two-step strategy. (i) The *TEC1* promoter region was amplified by PCR with primers TEC1-3 (ACGCGTCGACCATGGTTAAACAGGTATCAG AATTGTTG) and TEC1-4 (CTTCAGGCAAGAGTACGTTCTTCGCTGG)

TABLE 2. Plasmids used in this study

Plasmid	Description ^a	Reference
YCplac33	<i>URA3</i> -marked centromere vector	13
YEplac195	<i>URA3</i> -marked 2 μ m vector	13
YCplac111	<i>LEU2</i> -marked centromere vector	13
pRS315	<i>LEU2</i> -marked centromere vector	49
YEplac181	<i>LEU2</i> -marked 2 μ m vector	13
YEp356R	<i>URA3</i> -marker 2 μ m vector for <i>lacZ</i> fusions	37
pME2044	3.1-kb fragment containing <i>TEC1</i> in YCplac33	This work
pME2045	3.1-kb fragment containing <i>TEC1</i> in YCplac111	This work
pME2047	3.1-kb fragment containing <i>TEC1</i> in YEplac181	This work
pME2049	2.5-kb <i>GALI1(p)::TEC1</i> fusion in pRS315	This work
pME2071	2.5-kb <i>GALI1(p)::TEC1</i> fusion in pRS316	36
pME2077	<i>tec1-102</i> in YCplac33	This work
pME2083	<i>tec1-103</i> in YCplac33	This work
pME2085	<i>tec1-104</i> in YCplac33	This work
pME2086	<i>tec1-105</i> in YCplac33	This work
pME2102	<i>tec1-201</i> in YCplac33	This work
pME2096	<i>tec1-202</i> in YCplac33	This work
pME2101	<i>tec1-203</i> in YCplac33	This work
pME2103	<i>tec1-204</i> in YCplac33	This work
pME2290	<i>tec1-102</i> in YCplac111	This work
pME2291	<i>tec1-105</i> in YCplac111	This work
pME2293	<i>tec1-201</i> in YCplac111	This work
pME2292	<i>tec1-202</i> in YCplac111	This work
pME2068	<i>TEC1(p)::TEC1</i> in YCplac33	This work
pME2289	<i>TEC1(p)::TEC1</i> in YCplac111	This work
pME2294	<i>TEC1(p)::TEC1</i> in YEplac181	This work
pME2279	<i>TEC1(p)::myc³-TEC1</i> fusion in YCplac33	This work
pME2295	<i>TEC1(p)::myc⁶-TEC1</i> fusion in YCplac33	This work
pME2280	<i>TEC1(p)::myc³-TEC1</i> fusion in YEplac195	This work
pME2296	<i>TEC1(p)::myc⁶-TEC1</i> fusion in YEplac195	This work
pME2281	<i>TEC1(p)::myc³-tec1-102</i> fusion in YEplac195	This work
pME2282	<i>TEC1(p)::myc³-tec1-103</i> fusion in YEplac195	This work
pME2283	<i>TEC1(p)::myc³-tec1-104</i> fusion in YEplac195	This work
pME2284	<i>TEC1(p)::myc³-tec1-105</i> fusion in YEplac195	This work
pME2287	<i>TEC1(p)::myc³-tec1-201</i> fusion in YEplac195	This work
pME2285	<i>TEC1(p)::myc³-tec1-202</i> fusion in YEplac195	This work
pME2286	<i>TEC1(p)::myc³-tec1-203</i> fusion in YEplac195	This work
pME2288	<i>TEC1(p)::myc³-tec1-204</i> fusion in YEplac195	This work
pME2043	3.5-kb <i>TEC1(p)::abaA</i> fusion in YCplac33	This work
pME2046	3.5-kb <i>TEC1(p)::abaA</i> fusion in YCplac111	This work
pME2048	3.5-kb <i>TEC1(p)::abaA</i> fusion in YEplac181	This work
pME2050	3.1-kb <i>GALI1(p)::abaA</i> fusion in YCplac111	This work
pME2070	3.1-kb <i>GALI1(p)::abaA</i> fusion in pRS316	This work
pME2300	<i>TEC1-lacZ</i> fusion in YCplac33	This work
pME2065	<i>TEC1-lacZ</i> fusion in YEp356R	This work
B3782	<i>FLO11-lacZ</i> fusion in YEp356R	46
pME2064	<i>PGU1-lacZ</i> fusion in YEp356R	32
pLI4	<i>CYC1-lacZ</i> fusion in <i>URA3</i> -marked integrative vector	48
pME1108	pLI4 with <i>CYC1(ΔUAS)-lacZ</i>	This work
pME2051	pLI4 with <i>TCS^{fw}-CYC1-lacZ</i>	This work
pME2052	pLI4 with <i>TCS^{bw}-CYC1-lacZ</i>	This work
pME2056	pLI4 with <i>TCS^{fw}-6-TCS^{bw}-CYC1-lacZ</i>	This work
pME2055	pLI4 with <i>TCS^{fw}-11-TCS^{bw}-CYC1-lacZ</i>	This work
pME2053	pLI4 with <i>TCS^{fw}-14-TCS^{bw}-CYC1-lacZ</i>	This work
pME2057	pLI4 with <i>TCS^{fw}-13-TCS^{bw}-CYC1-lacZ</i>	This work
pME2058	pLI4 with <i>TCS^{fw}-13-TCS^{bw}-13-TCS^{bw}-CYC1-lacZ</i>	This work
pME2066	pLI4 with <i>TCS^{fw}-14-PRE^{fw}-CYC1-lacZ</i>	This work
ptec1 Δ ::HIS3	Cassette for full deletion of <i>TEC1</i> ORF	36
pste12 Δ ::TRP1	Cassette for full deletion of <i>STE12</i> ORF	This work

^a *TCS^{fw}* indicates forward orientation of TCS element, and *TCS^{bw}* marks backward orientation with respect to the orientation of the TCS element in *FRE(Ty1)* (31). *TCS^{fw}-14-PRE^{fw}* corresponds to the sequence of *FRE(Ty1)* (31).

and plasmid B3366 (34) as the template, yielding a 1.05-kb PCR product that was digested with *PstI* and *SalI* and inserted into plasmid YCplac33 to obtain plasmid YCplac33-*TEC1(p)*. (ii) A 2.1-kb fragment containing the *TEC1* open reading frame (ORF) was amplified from B3366 by using primers TEC1-5 (ACGCGTC GACAGTCTAAAGAAGACGACTTTGGCAAGG) and TEC1-6 (CGCG GATCCGGCCCCGACTTGAATGATTTTCAAGGTAGG), introducing a *SalI* site in front of the second codon of *TEC1* and a *BamHI* site 650 bp downstream

of the TAA stop codon. This fragment was inserted into the *SalI* and *BamHI* sites of YCplac33-*TEC1(p)* from step i to obtain plasmid pME2068, carrying a functional *TEC1(p)::TEC1* gene with a *SalI* restriction site after the ATG start codon. Plasmids pME2289 and pME2294 were obtained by subcloning of the *TEC1(p)::TEC1* cassette from pME2068 into the *PstI* and *BamHI* sites of YCplac111 and YCplac181, respectively. Plasmids pME2279 and pME2280, both expressing a triple myc epitope-tagged version of *Tec1p* under the control of the *TEC1* promoter, were obtained by the following cloning strategy. (i) A 3.1-kb *PstI-BamHI* fragment containing *TEC1(p)::TEC1* was subcloned from pME2068 into YEplac195. (ii) A synthetic *BglII* linker was ligated into the *SalI* site following the *TEC1* translational start codon. (iii) Finally, a 120-bp *BamHI* fragment carrying the triple myc epitope (*myc³*) was inserted into the *BglII* site to yield plasmid pME2280. The complete *TEC1(p)::myc³-TEC1* cassette was isolated by *PstI-SmaI* digestion and inserted into YCplac33 to yield pME2279. In addition, plasmids pME2295 and pME2296 were constructed, which differ from pME2280 and pME2279 only by the copy number of myc epitope tags. Plasmid pME2295 consists of a *TEC1(p)::myc⁶-TEC1* cassette in YCplac33, and plasmid pME2296 carries the same cassette in YEplac195. Plasmids carrying the different *tec1* mutant alleles were identified in a mutant allele library screen (see below). A subset of these *tec1* alleles were subcloned as 3.1-kb *PstI-BamHI* fragments into YCplac111 to obtain plasmids pME2290 to pME2293. The different *tec1* mutant alleles were myc epitope tagged by isolation of 2.1-kb *SalI-BamHI* fragments from the respective plasmids and exchange for the corresponding wild-type *TEC1* fragment in plasmids pME2280 and pME2279 to yield plasmids pME2281 to pME2288.

For expression of *A. nidulans*-derived *abaA* in *S. cerevisiae*, the *abaA* ORF was placed behind the *S. cerevisiae* *TEC1* promoter by using the following cloning strategy. (i) The intronless *abaA*-ORF was amplified by PCR from plasmid pAA35 (2) by using primers ABAA-1 (ACGCGTCGACGCTACTGACTGGC AACCCGAGTGTATGG) and ABAA-2 (ACGCGTCGACCTAGACAGCCT CAACCGAGTATGTTTC), introducing *SalI* sites at both ends. The resulting 2.4-kb PCR fragment was placed downstream of the *TEC1* promoter by insertion into the *SalI* site of YCplac33-*TEC1(p)* to yield pME2043. The whole *TEC1(p)::abaA* cassette of pME2043 was released by *PstI-SmaI* digestion and inserted into YCplac111 and YEplac181 to yield pME2046 and pME2048, respectively. Plasmids pME2050 and pME2070 were obtained by subcloning of a 3.1-kb *GALI1(p)::abaA* expression cassette from plasmid pAA35 (2) into YCplac111 and pRS316, respectively.

Plasmid pME1108 was constructed by deletion of a 430-bp *XhoI* fragment containing the *CYC1* UAS of plasmid pLI4 (48). Plasmids pME2051 to pME2058, carrying various combinations of TCS elements upstream of the *CYC1-lacZ* reporter gene, were constructed by substituting the *XhoI* fragment of pLI4 for one or several copies of a synthetic linker containing a single TCS element and *XhoI*-cohesive ends. The linker was prepared by annealing of primers TCS1 (TCGAGTCACATTCCTCTGTC [the TCS element is underlined]) and TCS2 (TCGAGCAGAGAATGTGAC [the reverse complement of the TCS element is underlined]). The number and orientation of the inserted TCS elements were determined by DNA sequence analysis (see Fig. 5). The integrative *FRE(Ty1)-lacZ* reporter plasmid pME2066 was obtained by subcloning a *SalI-BamHI* fragment from plasmid *FRE(Ty1)::lacZ* (31) into pLI4. The *TEC1-lacZ* reporter plasmid pME2065 was constructed by subcloning of the *TEC1* promoter as a 1.05-kb *PstI-SalI* fragment from pME2041 into YEp356R (37), and plasmid pME2300 was obtained by subcloning the *TEC1-lacZ* expression cassette from pME2065 into YCplac33.

Library of *TEC1* mutants. *TEC1* was mutagenized by PCR amplification of a 2.2-kb fragment of pME2068 containing the *TEC1* ORF by using *Taq* DNA polymerase in the presence of 0.24 mM MnCl₂. The resulting DNA was digested with *SalI* and *BamHI* and exchanged for the corresponding *SalI-BamHI* fragment in pME2068, yielding a library of more than 20,000 independent recombinants. Following identification of mutants (see below), *TEC1* alleles were sequenced by using the ABI Prism Big Dye terminator sequencing kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany).

Screen for *TEC1* invasive-growth mutants. For isolation of mutants with altered invasive growth and *FRE-lacZ* reporter induction, strain RH2499 carrying a chromosomal deletion of *TEC1* and an integrated *FRE(Ty1)::lacZ* reporter was transformed with the *TEC1* mutant library described above. A pool of approximately 30,000 transformants was plated on solid medium lacking uracil (SC-Ura medium) at a density of ~500 colonies per plate. Invasive-growth mutants were isolated by employing an invasive-growth test (44), and expression of the *FRE(Ty1)::lacZ* reporter gene was measured by a qualitative filter assay (4). Initial mutant phenotypes were confirmed by isolation of the *TEC1*-containing plasmids and reintroduction into the parental strain.

Pseudohyphal-development assay. Qualitative assays for pseudohyphal development were performed as described previously (33). After 3 days of growth on solid synthetic low-ammonium medium, pseudohyphal colonies were viewed with a Zeiss Axiovert microscope and photographed with a Kappa DX30 digital camera and the Kappa ImageBase software (Kappa Opto-Electronics, Gleichen, Germany).

β -Galactosidase assays. Strains carrying plasmid-borne or integrated *lacZ* reporters were grown in selective liquid SC medium to exponential growth phase, and extracts were prepared and assayed for β -galactosidase activity as described previously (33). β -Galactosidase specific activity was normalized to the total protein in each extract and equals (optical density at 420 nm \times 1.7)/(0.0045 \times protein concentration \times extract volume \times time). Assays were performed on at least three independent transformants, and the mean value is presented. Standard deviations did not exceed 20%.

Northern blot analysis. Total RNAs were prepared from exponentially growing liquid cultures as described earlier (6). Total RNAs were separated on a 1.4% agarose gel containing 3% formaldehyde and transferred onto nylon membranes as described earlier (35). *TEC1*, *FLO11*, and *ACT1* transcripts were detected by using gene-specific, 32 P-radiolabeled DNA probes. Hybridizing signals were quantified with a BAS-1500 Phospho-Imaging scanner (Fuji, Tokyo, Japan).

Protein analysis. Yeast strains harboring plasmids encoding myc-Tec1p were grown to exponential phase in liquid SC medium. Preparation of total cell extracts and subsequent Western blot analysis were performed essentially as previously described (45). myc-Tec1p fusion proteins were detected by enhanced-chemiluminescence technology (Amersham, Buckinghamshire, United Kingdom) after incubation of nitrocellulose membranes with mouse anti-myc monoclonal antibodies (9E10) together with a peroxidase-coupled goat anti-mouse immunoglobulin G secondary antibody (Dianova, Hamburg, Germany). For detection of Cdc42p, the membranes were incubated with polyclonal anti-Cdc42p antibodies and a peroxidase-coupled goat anti-rabbit immunoglobulin G secondary antibody (Dianova). Tec1p and Cdc42p signals were quantified with a scanner and Molecular Analyst software (Bio-Rad, Munich, Germany).

Indirect immunofluorescence microscopy. Yeast strains harboring plasmids encoding myc-Tec1p were cultured to exponential growth phase in liquid yeast nitrogen base medium supplemented with appropriate amino acids. Cells were harvested from 1 ml of the cultures by centrifugation and fixed in 3.7% formaldehyde. Spheroblasts were prepared as described previously (42). 4',6'-Diamidino-2-phenylindole (DAPI) staining and mouse anti-myc monoclonal antibodies (9E10), together with an Alexa 488-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, Oreg.), were used for visualization of nuclei and myc epitope-tagged proteins, respectively. Cells were viewed on a Zeiss Axiovert microscope by either differential interference contrast microscopy or fluorescence microscopy using standard DAPI and fluorescein isothiocyanate filter sets. Cells were photographed with a Xillix Microimager digital camera and Improvision Openlab software (Improvision, Coventry, United Kingdom).

RESULTS

Tec1p and AbaAp induce haploid invasive growth and pseudohyphal development in the absence of Ste12p. We tested whether Tec1p is able to induce haploid invasive growth and diploid pseudohyphal development of *S. cerevisiae* in the absence of Ste12p. Previous work had shown that the Tec1p homologue AbaAp from *A. nidulans* induces *S. cerevisiae* pseudohyphal development in strains lacking *STE12* but only when expressed from the highly inducible *GAL1* promoter (12). We expressed *TEC1* and *abaA* from the endogenous *TEC1* promoter on either a low-copy (CEN) or a high-copy (2μ m) plasmid in both haploid and diploid *tec1* Δ and *tec1* Δ *ste12* Δ mutant strains. In addition, both genes were expressed from the *GAL1* promoter. Haploid strains were assayed for invasive growth by a wash test (Fig. 1), and diploid pseudohyphal development was measured by growth on nitrogen starvation medium (Fig. 2).

As expected, haploid *tec1* Δ and *tec1* Δ *ste12* Δ mutant strains failed to grow invasively when harboring the empty vectors as control plasmids (Fig. 1). Expression of *TEC1* from the low-copy plasmid was sufficient to restore invasive growth in the *tec1* Δ strain but not in the *tec1* Δ *ste12* Δ background. However,

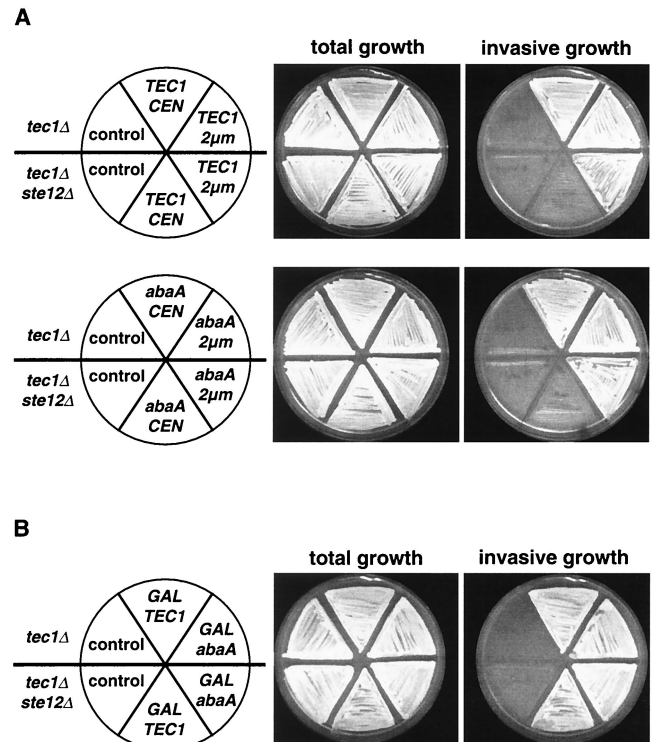


FIG. 1. Haploid invasive growth of yeast strains expressing *TEC1* or *A. nidulans* *abaA* driven by the *TEC1* promoter. (A) Haploid strains RH2500 (*tec1* Δ) and RH2501 (*tec1* Δ *ste12* Δ) carrying plasmid YCplac111 (control), pME2045 (*TEC1* CEN), pME2047 (*TEC1* 2μ m), pME2046 (*abaA* CEN), or pME2048 (*abaA* 2μ m) were grown on SC-Leu medium containing 2% glucose for 4 days. Plates were photographed before (total growth) and after (invasive growth) cells were washed off the agar surface. (B) Haploid strains RH2500 (*tec1* Δ) and RH2501 (*tec1* Δ *ste12* Δ) carrying plasmid YCplac111 (control), pME2049 (*GAL*-*TEC1*), or pME2050 (*GAL*-*abaA*) were grown on SC-Leu medium containing 2% galactose to induce expression from the *GAL1* promoter. Invasive growth was measured as described for panel A.

defective agar invasion of the *tec1* Δ *ste12* Δ strain was fully restored when *TEC1* was expressed either from a high-copy plasmid (Fig. 1A) or from the *GAL1* promoter (Fig. 1B). Identical results were obtained for strains expressing AbaAp instead of Tec1p. Expression of a single copy of *abaA* driven by the *TEC1* promoter restored agar invasion in the *tec1* Δ strain but not in the *tec1* Δ *ste12* Δ double mutant. As found for *TEC1*, defective invasive growth of the *tec1* Δ *ste12* Δ strain was suppressed by expression of *abaA* under control of the *TEC1* promoter from a high-copy plasmid or when driven by the *GAL1* promoter (Fig. 1A and B). These experiments show that both Tec1p and AbaAp can induce haploid invasive growth in the absence of Ste12p.

Pseudohyphal-development assays of diploid strains led to results similar to those obtained for invasive growth in haploids. Pseudohyphal growth was virtually absent in diploid *tec1* Δ /*tec1* Δ and *tec1* Δ /*tec1* Δ *ste12* Δ /*ste12* Δ mutant strains carrying control plasmids. Low-copy expression of *TEC1* restored defective pseudohyphal growth of the *tec1* Δ /*tec1* Δ mutant but not of the *tec1* Δ /*tec1* Δ *ste12* Δ /*ste12* Δ double mutant. Again, pseudohyphal-growth defects of strains lacking *STE12* could be suppressed by expression of *TEC1* from the high-copy plas-

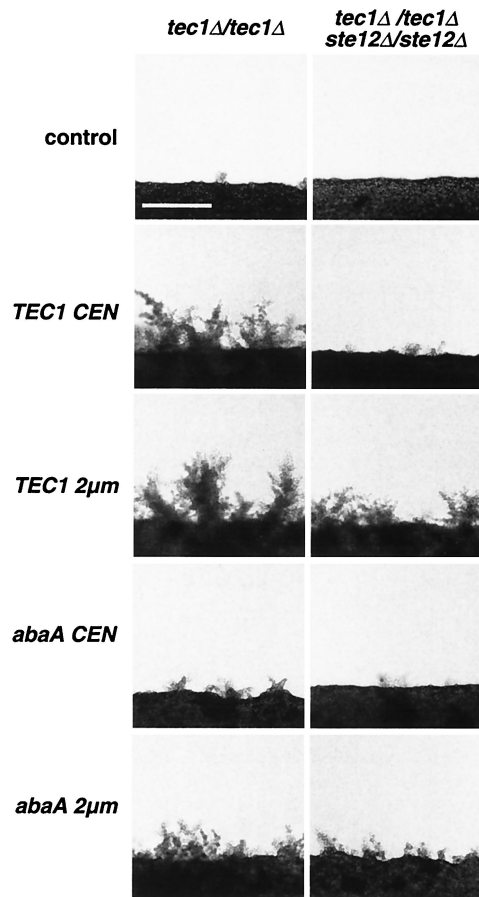


FIG. 2. Pseudohyphal growth of diploid yeast strains expressing *TEC1* or *A. nidulans abaA* driven by the *TEC1* promoter. Diploid strains RH2758 (*tec1Δ/tec1Δ*) and RH2759 (*tec1Δ/tec1Δ ste12Δ/ste12Δ*) carrying plasmid YCplac111 (control), pME2045 (*TEC1* CEN), pME2047 (*TEC1* 2 μ m), pME2046 (*abaA* CEN), or pME2048 (*abaA* 2 μ m) were transformed with YCplac33 to obtain uracil prototrophy. Transformants were grown on solid nitrogen starvation medium for 4 days before pseudohyphal development was visualized under a microscope and photographed. Bar, 100 μ m.

mid, although suppression was only partial compared to that of the *tec1Δ/tec1Δ* single mutant (Fig. 2). Low-copy expression of *abaA* driven by the *TEC1* promoter only weakly stimulated pseudohyphal growth in the *tec1Δ/tec1Δ* mutant. However, expression of *abaA* driven from a high-copy plasmid was sufficient to induce pseudohyphal growth in the *tec1Δ/tec1Δ* and *tec1Δ/tec1Δ ste12Δ/ste12Δ* mutants. In summary, Tec1p and AbaAp are able to at least partially induce pseudohyphal development in the absence of Ste12p.

Expression of *TEC1* requires Ste12p but is largely independent of Tec1p itself. Haploid yeast wild-type, *tec1Δ*, *ste12Δ*, and *tec1Δ ste12Δ* strains were constructed that express a myc epitope-tagged version of Tec1p (myc⁶-Tec1p) from either a low-copy or a high-copy plasmid to measure intracellular amounts of Tec1p. Strains were tested for invasive-growth behavior and found to be indistinguishable from strains expressing untagged Tec1p (data not shown), demonstrating that the epitope-tagged version is fully functional. When expressed from a low-copy plasmid, myc⁶-Tec1p levels were identical in

wild-type and *tec1Δ* strains (Fig. 3A). In contrast, myc⁶-Tec1p levels dropped roughly 20-fold in strains lacking *STE12* (*ste12Δ* and *tec1Δ ste12Δ*), which consequently were unable to grow invasively. Thus, Ste12p is required for expression of Tec1p to levels required for induction of invasive growth. When expressed from a high-copy plasmid, almost identical amounts of myc⁶-Tec1p were detectable in all strains, even in the *ste12Δ* mutants (Fig. 3A). Levels of myc⁶-Tec1p expressed from high-copy-number plasmids were not more than 2.7-fold higher than levels obtained by low-copy expression of myc⁶-Tec1p in wild-type or *tec1Δ* strains. Thus, expression of *TEC1* from the high-copy-number plasmid in *ste12Δ* mutant strains reflects a situation in which, in the absence of Ste12p, the amount of Tec1p is restored and therefore allows invasive growth.

Expression of a translational *TEC1-lacZ* fusion gene from low-copy and high-copy plasmids was measured in haploid wild-type and *tec1Δ*, *ste12Δ*, and *tec1Δ ste12Δ* mutant strains to quantify regulation of *TEC1* gene expression by Ste12p and by Tec1p itself. Previous studies had shown that *TEC1* transcript levels drop at least sixfold when Ste12p is absent and that activation of *TEC1* transcription by Ste12p is predominantly mediated by several PRE sites present in the *TEC1* promoter and only to a minor extent by its single FRE site (24, 40). We found that *TEC1* is not required for expression of a *TEC1-lacZ* fusion gene expressed from the low-copy plasmid and only to a minor extent for expression of the high-copy version (Fig. 3B). This indicates that Tec1p does not greatly contribute to its own expression, a conclusion that is supported by the previous finding that high-copy expression of Tec1p induces its own expression not more than twofold (31). In contrast, expression of *TEC1-lacZ* was reduced between 4.5-fold (low-copy *TEC1-lacZ*) and 11-fold (high-copy *TEC1-lacZ*) when Ste12p was absent. High-copy expression of *TEC1-lacZ* in the *ste12Δ* background was only 3.8-fold higher than levels obtained by low-copy expression in strains carrying a functional *STE12* gene, corroborating the results obtained by measuring Tec1p protein levels (Fig. 3A). However, several differences between *TEC1-lacZ* expression and Tec1p protein levels were found. In *STE12*-carrying strains (wild type or *tec1Δ*), expression of *TEC1-lacZ* from the high-copy plasmid was 42-fold higher than from the low-copy version (Fig. 3B). In contrast, Tec1p protein levels in *STE12* strains are only 2.7-fold higher when *TEC1* is expressed from the high-copy plasmid than when it is expressed from the low-copy version (Fig. 3A). Furthermore, *TEC1-lacZ* expression dropped between 4.5- and 11-fold when *STE12* was deleted, whereas Tec1p protein levels decrease by a factor of 20 in the absence of Ste12p. These discrepancies could be explained by a difference in protein stability between Tec1p and β -galactosidase. In summary, Ste12p appears to control the expression of *TEC1* to a larger extent than Tec1p itself.

Tec1p enters the yeast nucleus without Ste12p. Intracellular localization of Tec1p has not been determined directly, although indirect evidence suggests that Tec1p is a nuclear protein (31). Here, subcellular localization of Tec1p was determined to analyze whether Ste12p is required for nuclear transport. Wild-type, *tec1Δ*, and *ste12Δ* strains that express myc epitope-tagged Tec1p from a low-copy or high-copy plasmid were used for indirect immunofluorescence microscopy. Specific myc⁶-Tec1p signals could be detected in the nuclei of

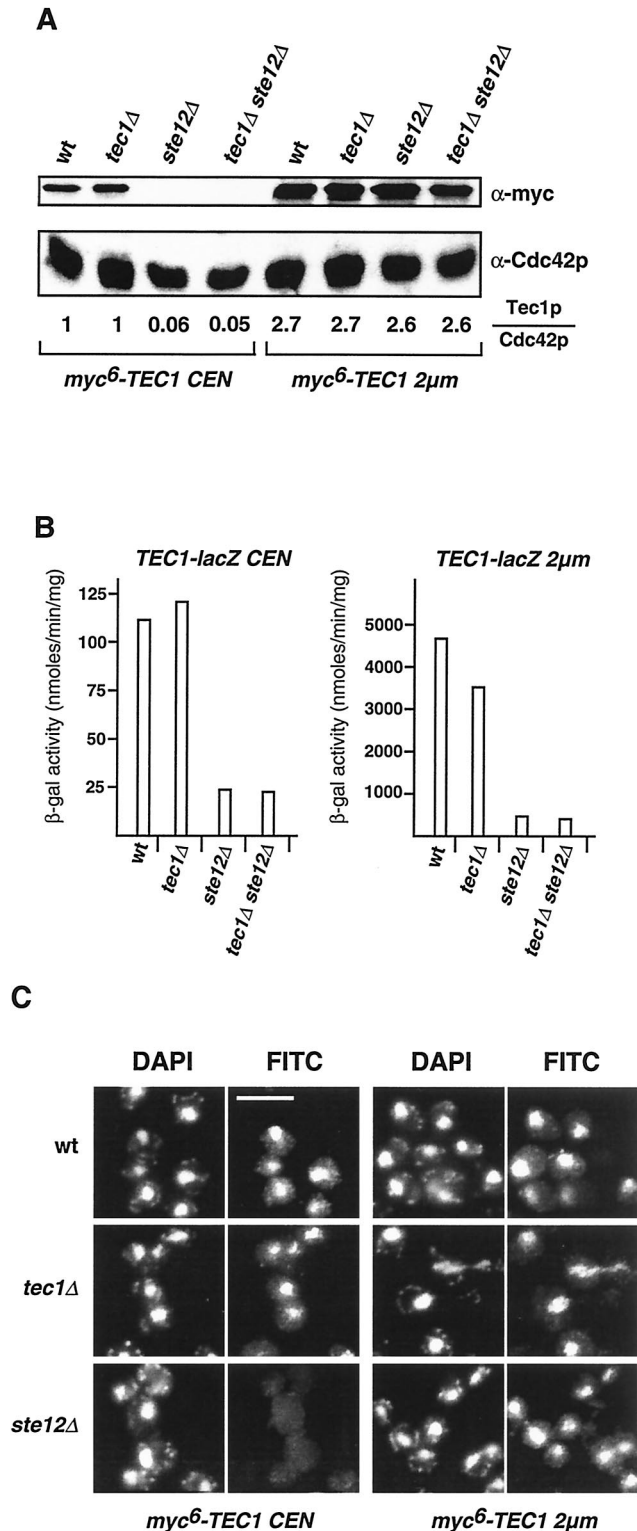


FIG. 3. Expression and localization of Tec1p in *S. cerevisiae*. (A) Expression of myc⁶-Tec1p. Protein extracts were prepared from haploid yeast strains RH2754 (wild type [wt]), RH2500 (*tec1Δ*), RH2755 (*ste12Δ*), and RH2501 (*tec1Δ ste12Δ*) expressing myc⁶-TEC1 on low-copy plasmid pME2295 (CEN) or high-copy plasmid pME2296 (2µm), and levels of myc⁶-Tec1p were determined by Western blot analysis with an anti-myc monoclonal antibody. As an internal control, expression levels of Cdc42p were measured in the same extracts by using an anti-Cdc42p polyclonal antibody (bottom). Relative Tec1p (Tec1p/

wild-type and *tec1Δ* strains with both low- and high-copy expression (Fig. 3C). In the *ste12Δ* strain, specific nuclear signals were absent when myc⁶-Tec1p was expressed from the low-copy plasmid, corroborating the results obtained by Western blot analysis (Fig. 3A). Importantly, nuclear localization of myc⁶-Tec1p was restored in the *ste12Δ* strain when expressed from the high-copy plasmid, suggesting that Tec1p is a nuclear protein that enters the nucleus even in the absence of Ste12p.

Tec1p and AbaAp activate expression of FLO11 and PGU1 in the absence of Ste12p. Our finding that Tec1p can induce invasive growth independently of Ste12p prompted us to measure expression of *FLO11* and *PGU1* in *ste12Δ* strains in which Tec1p levels had been restored by using high-copy-number plasmids. Expression of both the *FLO11* and *PGU1* genes is strongly reduced in the absence of either Tec1p or Ste12p (29, 32, 46). We reasoned that if expression of *FLO11* and *PGU1* could be activated in strains lacking Ste12p but expressing Tec1p at sufficient levels, activation of *FLO11* and *PGU1* by Tec1p would have to involve an Ste12p-independent control mechanism.

FLO11 transcript levels were measured in *tec1Δ* and *tec1Δ ste12Δ* strains expressing *TEC1* or *A. nidulans* derived *abaA* from either the endogenous yeast *TEC1* promoter on low- and high-copy plasmids or from the *GAL1* promoter (Fig. 4A). In the *tec1Δ* genetic background, introduction of single copies of either *TEC1* or *abaA* into yeast induced *FLO11* transcription 4.2-fold (*TEC1*) and 4.7-fold (*abaA*) compared to that in a control strain expressing no Tec1p. High-copy expression of *TEC1* or *abaA* or expression from the inducible *GAL1* promoter led to an increase in *FLO11* transcript levels of 4.9-fold (high-copy *TEC1*), 8.5-fold (high-copy *abaA*), 13-fold (*GAL1-TEC1*), or 5.6-fold (*GAL1-abaA*) compared to those in strains expressing a single copy of *TEC1*. Thus, expression of *FLO11* depends on the dosage of Tec1p and AbaAp. In the *tec1Δ ste12Δ* strain, introduction of a single copy of *TEC1* or *abaA* was not sufficient to activate normal *FLO11* expression. However, high-copy expression of Tec1p or AbaAp restored *FLO11* transcription to levels detected in the *tec1Δ* strain expressing single copies of *TEC1* or *abaA* (corresponding to a wild-type situation). Even stronger induction of *FLO11* transcription was measured for *GAL1-TEC1* (13-fold) and *GAL1-abaA* (5.1-fold). These results demonstrate that, in the absence of Ste12p, both Tec1p and AbaAp are able to activate expression of *FLO11* to levels sufficient for invasive growth.

Cdc42p) expression levels are shown in arbitrary units and were obtained by normalizing Tec1p signals to Cdc42p signals and to levels measured in strain RH2500 (*tec1Δ*) expressing *TEC1* from a low-copy plasmid. (B) *TEC1-lacZ* expression levels. β-Galactosidase (β-gal) specific activity was measured in haploid strains carrying *TEC1-lacZ* on low-copy plasmid pME2300 (CEN) or high-copy plasmid pME2065 (2µm) and in the presence or absence of *TEC1* and *STE12* in different combinations. Bars depict means of three independent measurements, with standard deviations not exceeding 15%. (C) Localization of myc⁶-Tec1p. Haploid strains RH2754 (wt), RH2500 (*tec1Δ*), and RH2755 (*ste12Δ*) expressing myc⁶-TEC1 on low-copy plasmid pME2295 (CEN) or high-copy plasmid pME2296 (2µm) were grown to exponential phase and prepared for an anti-myc immunofluorescence assay. Shown are representative cells that were viewed for nuclear DNA with DAPI imaging or for anti-myc immunofluorescence (FITC). Bar, 5 µm.

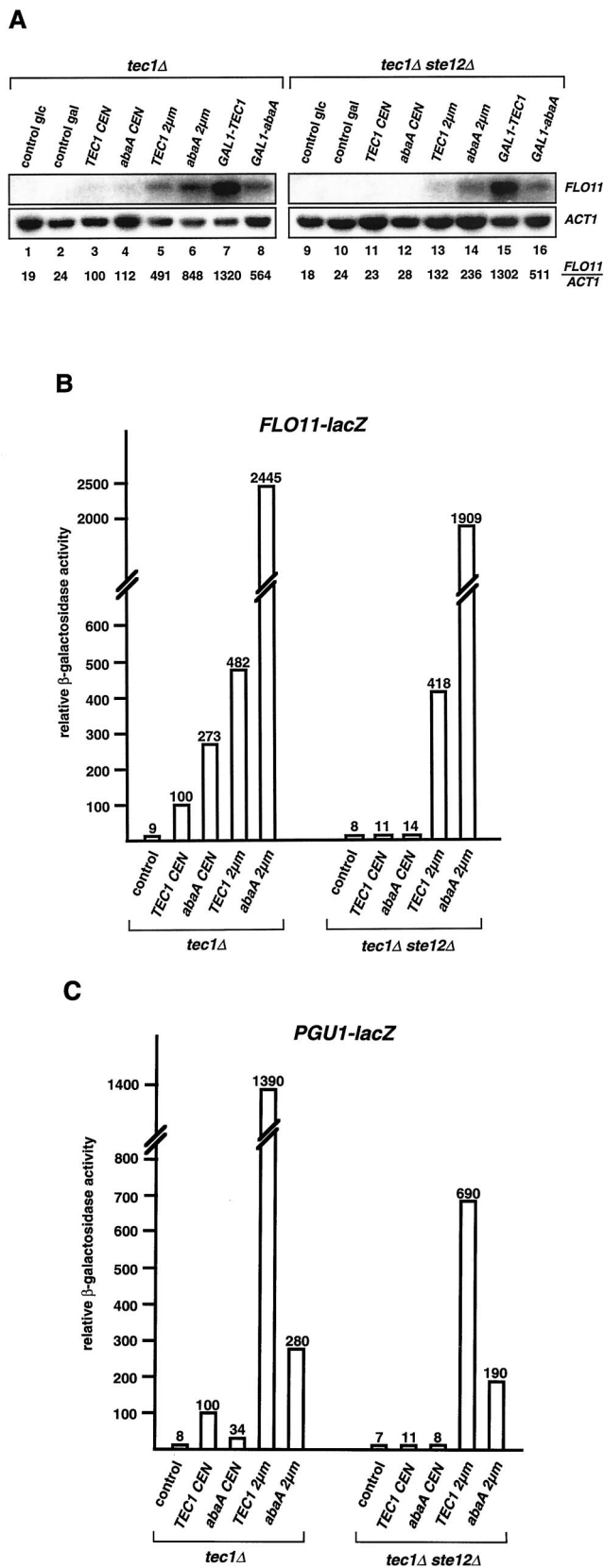


FIG. 4. *TEC1* and *abaA* activate expression of *FLO11* and *PGU1* in the absence of *STE12* in yeast. (A) *FLO11* transcript levels. Strains RH2500 (*tec1Δ*) and RH2501 (*tec1Δ ste12Δ*) carrying plasmid YCplac111

Results obtained by analysis of *FLO11* transcript levels were further corroborated by β -galactosidase assays with a *FLO11-lacZ* reporter gene (46) that was introduced into *tec1Δ* and *tec1Δ ste12Δ* yeast strains expressing *TEC1* and *abaA* at different levels (Fig. 4B). In the *tec1Δ* strain, single copies of *TEC1* or *abaA* increased *FLO11-lacZ* reporter activity 11.1-fold (*TEC1*) or 27.3-fold (*abaA*) in comparison with that of a control strain lacking *TEC1* (Fig. 4B). High-copy expression of *TEC1* induced the expression of *FLO11-lacZ* 4.8-fold compared with that of low-copy *TEC1*, whereas high-copy *abaA* led to 24.4-fold higher reporter activity. Again, a single copy of *TEC1* or *abaA* was not sufficient to activate *FLO11-lacZ* expression in the absence of *STE12* (*tec1Δ ste12Δ* background), whereas high-copy expression of both *TEC1* and *abaA* led to high levels of *FLO11-lacZ* reporter activity that were comparable to those measured in the presence of *STE12* (*tec1Δ* background).

Regulation of *PGU1* was measured by use of a *PGU1-lacZ* reporter gene (32) that was expressed in *tec1Δ* and *tec1Δ ste12Δ* strains containing no, single, or high numbers of copies of *TEC1* or *abaA* (Fig. 4C). Expression of *PGU1-lacZ* was stimulated 12.5-fold by the introduction of a single *TEC1* copy into the *tec1Δ* background and a further 13.9-fold by high-copy expression of *TEC1*. A 3.4-fold induction was obtained by a single copy, and a further 8.2-fold induction was obtained by high-copy *abaA*. In the absence of *Ste12p* (*tec1Δ ste12Δ* strain), a single copy of *TEC1* or *abaA* was not able to significantly stimulate *PGU1-lacZ* reporter activity beyond basal levels, but high-copy *TEC1* or *abaA* activated reporter expression 99-fold (*TEC1*) and 27-fold (*abaA*), respectively. In summary, Tec1p and AbaAp significantly activate expression of *FLO11* and *PGU1* in the absence of *Ste12p*, a fact that might explain why these transcription factors are able to induce invasive growth in *ste12Δ* strains.

Tec1p activates gene expression via TCS elements in the absence of *Ste12p*. The sole mechanism by which Tec1p has been proposed to activate gene expression is cooperative bind-

(control), pME2045 (*TEC1* CEN), pME2046 (*abaA* CEN), pME2047 (*TEC1* 2 μ m), pME2048 (*abaA* 2 μ m), pME2049 (*GAL1-TEC1*), or pME2050 (*GAL1-abaA*) were grown in SC-Leu medium containing 2% glucose (lanes 1, 3, 4, 5, 6, 9, 11, 12, 13, and 14) or 2% galactose (lanes 2, 7, 8, 10, 15, and 16) to exponential phase before total RNA was prepared and used for Northern analysis. *ACT1* gene expression served as an internal standard. Relative *FLO11* (*FLO11/ACT1*) expression levels were obtained by using a Phospho-Imaging scanner and normalizing *FLO11* expression measured in control strain RH2500 (*tec1Δ*) expressing *TEC1* from a low-copy plasmid (lane 3). (B) *FLO11-lacZ* expression levels. Strains described in panel A were transformed with plasmid B3782 carrying the *FLO11-lacZ* reporter (46) and grown to exponential phase before β -galactosidase specific activity was measured. Bars depict relative values normalized to the activity measured in strain RH2500 (*tec1Δ*) expressing *TEC1* from a CEN plasmid, which was set at 100. (C) *PGU1-lacZ* expression levels. β -Galactosidase activity was measured in strains carrying *PGU1-lacZ* reporter plasmid pME2064 (32) and expressing *TEC1* and *A. nidulans abaA* driven by the *TEC1* promoter at different levels. Activities are given as relative values normalized to that of strain RH2500 (*tec1Δ*) expressing *TEC1* from a CEN plasmid, which was defined as 100. The bars in panels B and C depict means of three independent measurements, with standard deviations not exceeding 20%.

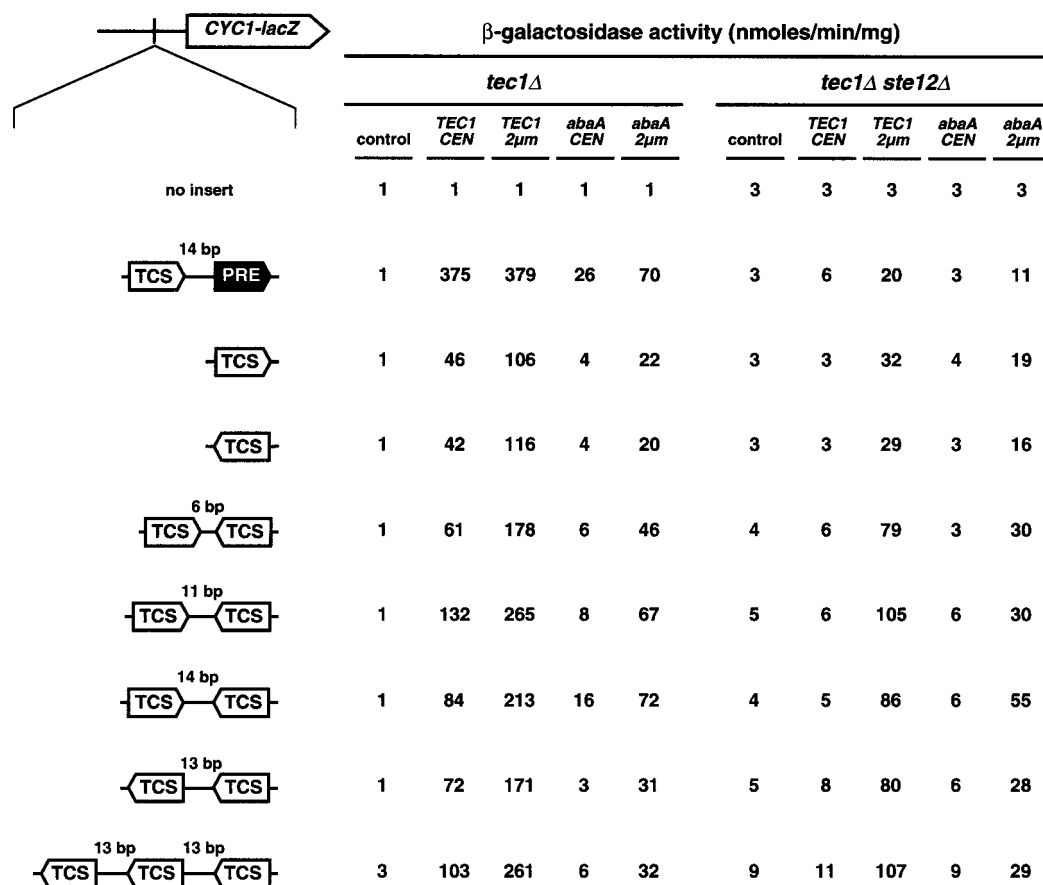


FIG. 5. Activation of FRE- and TCS-dependent gene expression by *TEC1*, *abaA*, and *STE12* in yeast. An enhancerless *CYC1-lacZ* fusion gene carrying no UAS element (no insert), FRE (TCS plus PRE), or TCS elements in different combinations was integrated as a single copy into the genomes of strains RH2500 (*tec1* Δ) and RH2501 (*tec1* Δ *ste12* Δ), and β -galactosidase specific activity was measured with *TEC1* and *abaA* being absent (control) or present at a low (CEN) or high (2 μ m) copy level. Activities are expressed in nanomoles of *o*-nitrophenyl- β -D-galactopyranoside hydrolyzed per minute times milligrams of protein and are means of four measurements with two independent transformants. The standard deviation was less than 20%.

ing together with Ste12p to FRE sites present in the promoter region of target genes (3, 31, 32). In contrast, AbaAp activates target gene expression by binding to repeated TCS elements (1). By a computer search, we found that the promoter regions of both *FLO11* and *PGU1* contain several TCS sites without neighboring PRE sites, as found in a typical FRE site. The *FLO11* promoter region contains four single TCS elements and a sequence composed of two TCS elements in close proximity, whereas the *PGU1* promoter region contains three single TCS sites in a different orientation but does not contain a typical FRE. This suggested that Tec1p might activate gene expression not only via FRE sites but also via TCS elements. To test this hypothesis, we constructed a series of TCS-driven reporter genes by insertion of TCS elements in different numbers and orientations upstream of an enhancerless *CYC1-lacZ* gene (16).

Expression of these reporters was measured in *tec1* Δ and *tec1* Δ *ste12* Δ yeast strains expressing *TEC1* at different levels and compared to that of *CYC1-lacZ* reporter genes containing either no UAS element or a single FRE site (Fig. 5). Without a functional UAS element, the *CYC1-lacZ* reporter could not be activated beyond basal levels under any condition. A single

FRE in front of *CYC1-lacZ* led to a 375-fold induction of reporter activity that was dependent on *TEC1* and *STE12*. In the presence of *STE12* alone (control plasmid in the *tec1* Δ strain), no activation of the FRE-driven reporter beyond basal levels was found, corroborating the finding that Tec1p and Ste12p cooperatively activate FRE-driven gene expression (31). However, *TEC1* alone was sufficient to activate the FRE-driven reporter up to sevenfold even without *STE12* (*TEC1* on a 2 μ m plasmid in the *tec1* Δ *ste12* Δ strain), suggesting that the TCS element present in the FRE site can be used by Tec1p, mediating significant activation of gene expression. This finding is in agreement with the fact that single TCS elements in a forward or backward orientation were sufficient to mediate at least 10-fold activation of the *CYC1-lacZ* reporter by Tec1p, even in the absence of Ste12p. In contrast to the FRE-reporter, activation mediated by TCS elements was Tec1p dosage dependent, because induction was 2- to 3-fold (up to 116-fold) higher in strains containing a 2- to 3-fold greater amount of Tec1p (*TEC1* expressed from a 2 μ m plasmid in a *tec1* Δ background) compared to activation (up to 46-fold) measured in strains expressing lower levels of Tec1p (*TEC1* expressed from a CEN plasmid; compare Fig. 3A). Dosage dependence was

also observed when *STE12* was absent. TCS reporters were not induced in *ste12Δ* strains expressing *TEC1* from low-copy plasmids (*TEC1* CEN), a finding that is explained by the fact that Ste12p is required for *TEC1* expression and, therefore, in these strains, only low levels of Tec1p can be detected by Western analysis (Fig. 3A). However, restoration of intracellular amounts of Tec1p (*TEC1* expressed from a 2 μ m plasmid in *tec1Δ ste12Δ* strains) also restored activation of the TCS-driven reporters (up to 10-fold) comparable to a wild-type situation. Double TCS elements led to activation of *CYCI-lacZ* reporter expression between 61- and 265-fold, depending on the spacing between the TCS elements and the Tec1p levels. Optimal activation was observed when spacing between TCS elements was 11 bp. Stimulation of gene expression was almost threefold better than that obtained by single TCS elements, pointing to a synergistic effect of the double element. In contrast, double TCS elements separated by 6 bp led to only 1.3-fold better activation (61 U) than single TCS elements (46 U). As found for single-TCS-driven reporters, activation mediated by double TCS elements was Tec1p dosage dependent. Again, restoration of Tec1p protein levels in *ste12Δ* strains by expression of *TEC1* from the 2 μ m plasmid led to activation of the double-TCS reporters comparable to that of the wild-type situation. We further tested a reporter construct composed of three TCS elements in a backward orientation, each separated by 13 bp. In comparison to the reporter with two backward-oriented TCS elements, this triple-TCS reporter could be further activated (261-fold versus 171-fold) when Tec1p was present at high levels.

In summary, these results demonstrate that Tec1p is able to activate gene expression not only via FREs (FRE control) but also via single or multiple TCS elements (TCS control). Interestingly, TCS control depends on Ste12p to some degree because we found a consistent two- to threefold drop in the expression of all TCS reporters in *ste12Δ* strains compared to *STE12* strains (which contain comparable levels of Tec1p). However, Tec1p alone is sufficient to significantly activate TCS-dependent gene expression up to 20-fold even in the absence of Ste12p.

FRE- and TCS-driven reporters were further tested for activation by *abaA*. *abaA* activated the FRE reporter 26-fold when expressed from the endogenous *TEC1* promoter on a low-copy plasmid. Interestingly, AbaAp appears to activate the FRE reporter cooperatively with Ste12p, because activation was up to 70-fold (2 μ m plasmid), whereas activation mediated by single TCS elements was not more than 22-fold. This suggests even greater similarity between the mechanisms of Tec1p and AbaAp function than previously assumed. Double-TCS-driven reporters were activated by AbaAp between 31- and 72-fold, depending on the spacing between the TCS elements. The best activation (72-fold) was found for double-TCS elements oriented as inverted repeats and separated by 14 bp. This finding is in agreement with an earlier study showing that inverted TCS repeats with a 13-bp spacing are optimal for activation (1). As found for Tec1p, TCS-mediated activation by AbaAp was functional even in strains lacking Ste12p.

The C-terminal region of Tec1p is required for haploid invasive growth and TCS-mediated reporter gene expression but is dispensable for FRE-driven gene expression. We isolated novel variants of the *TEC1* gene to characterize func-

TABLE 3. Invasion-defective and hyperinvasive *TEC1* mutants

Class and allele	Mutation(s)	Invasive growth ^a	Fold <i>FRE-lacZ</i> expression ^b
Control			
<i>tec1Δ</i>	Full deletion	–	<1
<i>TEC1</i>	None	+++	100
Class I			
<i>tec1-102</i>	Δ(K281-Y486)	+	67
<i>tec1-103</i>	E222G, Δ(Y257-Y486)	+	89
<i>tec1-104</i>	F93L, Δ(P335-Y486)	+	104
<i>tec1-105</i>	Δ(I353-Y486)	+	98
Class II			
<i>tec1-201</i>	T273M	++++	166
<i>tec1-202</i>	P274S	++++	157
<i>tec1-203</i>	K154R, S453P	++++	127
<i>tec1-204</i>	K154R, S262G	++++	105

^a Invasive growth was measured by a plate-washing assay and quantified as described in reference 34.

^b Induction of an *FRE-lacZ* reporter by the different *TEC1* alleles was measured in strain RH2499. The values shown are relative β -galactosidase activities normalized to the activity obtained with wild-type *TEC1*, which was set at 100.

tional domains of the Tec1p protein. A library of PCR-mutagenized *TEC1* alleles was created and introduced into a haploid *tec1Δ* yeast strain that carried a chromosomally integrated *FRE-lacZ* reporter gene. Resulting transformants were screened for invasive growth and *FRE-lacZ* reporter activity, yielding two classes of mutants (Table 3 and Fig. 6A). Class I mutants were defective for invasive growth but still promoted induction of the *FRE-lacZ* reporter. Class II mutants were enhanced for both invasive growth and *FRE-lacZ* expression. No class of mutants could be isolated that grew invasively but failed to activate *FRE-lacZ*. Mutants being suppressed for both phenotypes were excluded. Sequence analysis revealed that each of the class I mutant alleles encoded a C-terminally truncated protein and that all of the class II alleles coded for proteins with single or double amino acid substitutions (Table 3).

A triple-myc epitope tag was inserted just after the start codons of all *TEC1* mutant genes to further characterize mutant proteins. No phenotypic differences were detected between the epitope-tagged and nontagged versions (data not shown). *TEC1* mRNA and Tec1p protein levels were measured in all mutants to exclude the possibility that reduced expression or stability of Tec1p mutant proteins might account for the phenotypes observed (Fig. 6B and C). No significant decrease in mRNA levels was found for any of the *TEC1* mutant alleles. However, we detected increases in the transcript levels of *tec1-102* (2.5-fold), *tec1-103* (1.9-fold), *tec1-201* (1.6-fold), and *tec1-202* (1.5-fold). Levels of the Tec1-102p, Tec1-103p, Tec1-104p, and Tec1-105p proteins were markedly higher than that of the wild-type protein, excluding the possibility that low expression of these variants was responsible for the reduced invasiveness of the corresponding mutant strains. In contrast, reduced levels of the Tec1-203p and Tec1-204p proteins were detected, ruling out the possibility that increased expression of these mutant forms was the cause of the enhanced invasive growth of these class II mutants.

FLO11 transcripts levels were measured in the different *TEC1* mutant strains (Fig. 6B). All strains expressing class I

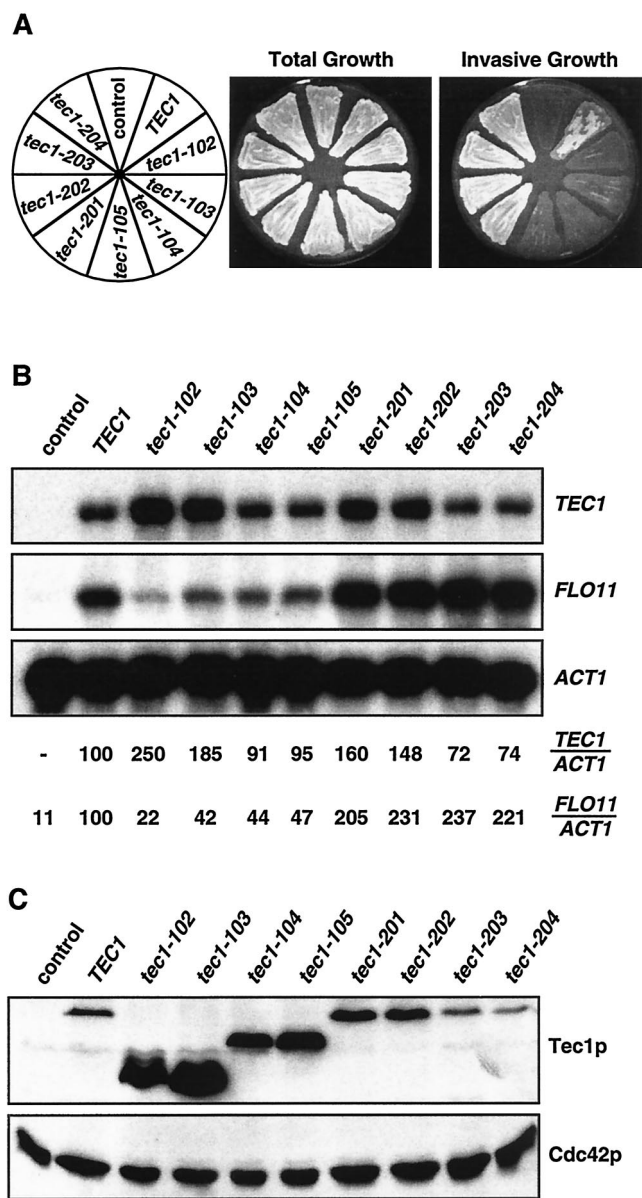


FIG. 6. Characterization of novel yeast *TEC1* alleles. (A) Regulation of haploid invasive growth. Strain RH2499 (*tec1Δ*) carrying a control plasmid, wild-type *TEC1*, or different *TEC1* mutant alleles on low-copy plasmids was patched on SC-Leu-Ura medium and grown for 4 days before the plate was photographed before (total growth) and after (invasive growth) cells were washed off the agar surface. (B) Expression of *FLO11* depending on different *TEC1* alleles. Shown are transcript levels of *TEC1*, *FLO11*, and *ACT1* measured in strain RH2499 (*tec1Δ*) expressing different *TEC1* alleles. *ACT1* transcripts served as an internal standard. Relative expression levels of *TEC1* alleles (*TEC1/ACT1*) and *FLO11* (*FLO11/TEC1*) are indicated below and were obtained with a Phospho-Imaging scanner, followed by normalization of *TEC1* and *FLO11* transcript levels to *ACT1* levels and to a strain expressing wild-type *TEC1*. (C) Western blot analysis. Shown are steady-state levels of the various myc-tagged Tec1p proteins obtained by expression of the indicated *TEC1* alleles in strain RH2499. Proteins were detected by using an anti-myc monoclonal antibody, and expression of Cdc42p was measured in the same extracts as an internal control.

mutant alleles showed a significant decrease in *FLO11* expression, with levels varying between 22 and 47% with respect to the wild-type control, whereas values between 205 and 237% were obtained with strains expressing the class II alleles. Thus, the degree of invasive growth correlates well with expression of *FLO11* in both classes of *TEC1* mutants.

Two *TEC1*-encoded mutant proteins each from classes I and II were further tested for activation of four different *TCS-lacZ* reporters and compared to *FRE-lacZ* reporter activation (Fig. 7). From class I, the severely truncated Tec1-102p mutant protein (Δ K281-Y486) was almost completely deficient in the activation of any of the *TCS-lacZ* reporters, with values reaching not more than 16% of those of the wild-type control. In contrast, Tec1-102p was still able to efficiently activate expression of *FRE-lacZ* to levels corresponding to 67% of the level achieved by wild-type Tec1p. The less severely truncated Tec1-105p variant (Δ I353-Y486) activated *FRE-lacZ* indistinguishably from wild-type Tec1p, whereas expression of the single *TCS-lacZ* reporters dropped to 41% (forward orientation of TCS) or 45% (backward orientation). Expression of double-*TCS-lacZ* reporters by Tec1-105p was found to be reduced less significantly, with values of up to 83% of the wild-type control. From class II, Tec1-201p and Tec1-202p strongly activated all *TCS-lacZ* reporters between 3.0- and 4.6-fold in comparison to wild-type Tec1p. In contrast, these variants stimulated *FRE-lacZ* no more than 1.6-fold.

In summary, these data suggest that distinct domains of Tec1p are required for activation of gene expression mediated by either TCS or FRE sites (Fig. 8). The N-terminal region of Tec1p appears to be sufficient for transcriptional activation via FRE but not via TCS sites. TCS-mediated activation by Tec1p additionally requires the C-terminal part of the protein.

DISCUSSION

Ste12p and Tec1p regulate gene expression and cellular development by several distinct mechanisms. The role of the TEA/ATTS family transcription factor Tec1p from *S. cerevisiae* in the regulation of gene expression and cellular development was investigated. Tec1p was previously thought to regulate these processes primarily by cooperative binding, together with Ste12p, to FRE sites present in the promoter region of target genes and of *TEC1* itself. This combinatorial model of Tec1p regulation is based on the observation that strains lacking either *TEC1* or *STE12* or both are equally suppressed for haploid invasive and diploid pseudohyphal growth (12, 27, 31, 34, 36, 44). The finding that FRE enhancer elements are bound in vitro by Tec1p and Ste12p in a cooperative manner further supports this model (31). The assumption that combinatorial control is the sole mechanism of Tec1p-regulated processes implies that the presence of either Tec1p or Ste12p alone is not sufficient to activate target gene expression and development. However, this prediction has not been tested experimentally prior to our study. Here, we have shown that strains containing sufficient amounts of Tec1p but lacking Ste12p are able to activate natural target genes and reporter genes that are driven by TCS elements. As a consequence, these strains undergo significant cellular development, although they do not contain any Ste12p. We further present evidence that Ste12p controls expression of *TEC1* to a larger extent than Tec1p itself, sug-

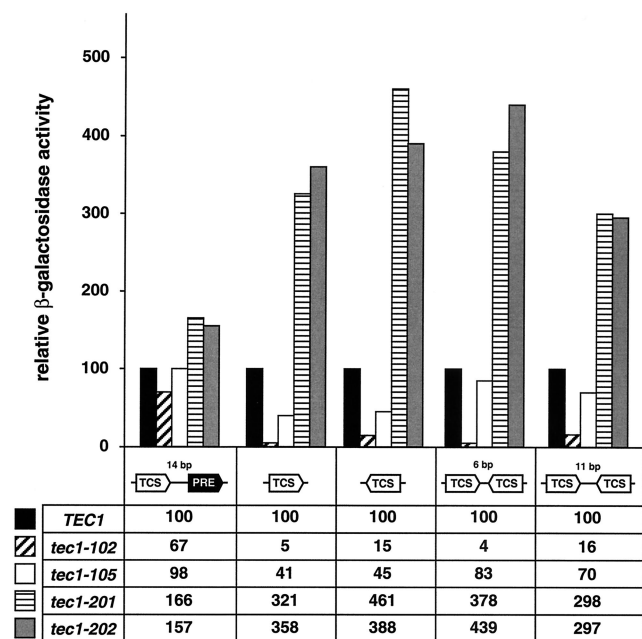


FIG. 7. Activation of FRE- and TCS-dependent *CYC1-lacZ* reporter genes by *TEC1* mutant alleles in yeast. Wild-type *TEC1* and *tec1-102*, *tec1-105*, *tec1-201*, and *tec1-202* mutant alleles were expressed in *tec1Δ* mutant strains carrying a *CYC1-lacZ* fusion gene driven by an FRE (TCS plus PRE) or TCS elements in different combinations, and strains were assayed for β -galactosidase specific activity. Bars and values represent relative activities normalized to the value obtained by expression of wild-type *TEC1* (black bars), which was set at 100. All values are means of three independent measurements. The standard deviation was less than 20%.

gesting the involvement of mechanisms other than combinatorial control. Our data suggest that Ste12p and Tec1p control gene expression and cellular development by several distinct mechanisms (Fig. 9). We propose a model in which Ste12p regulates expression of *TEC1* by both combinatorial control together with Tec1p via FRE sites and without Tec1p by acting via clustered PRE sites present in the *TEC1* promoter. In turn, Tec1p regulates gene expression by two distinct mechanisms, FRE control in combination with Ste12p and TCS control that is operative even without Ste12p.

TCS control is an efficient mechanism for activation of developmental target genes by Tec1p. A central finding of our study is that single TCS elements inserted upstream of a reporter gene are sufficient to mediate activation of gene expression by Tec1p. Activation is up to 100-fold and independent of the orientation of the TCS. This finding correlates well with the distribution and arrangement of TCS elements found in natural *S. cerevisiae* promoter regions. In silico analysis of the complete *S. cerevisiae* genome reveals a group of more than 100 genes that contain at least four TCS elements in their promoter regions (our unpublished results). Most of these TCS elements are arranged individually; that is, they are separated from each other by at least 20 bp and are not neighbored by an Ste12p-binding site. Remarkably, this group includes *FLO11*, *FLO8*, *PHD1*, *DFG10*, *CLN1*, *CDC25*, and *PGU1*, which are all genes that have previously been implicated in invasive and pseudohyphal growth (14, 15, 28–30, 32, 34, 46). Here, we have

shown that two representatives of these genes, *FLO11* and *PGU1*, are activated by Tec1p even when Ste12p is absent. This suggests that Tec1p, much like other TEA/ATTS family transcription factors, can activate target genes and cellular development also without Ste12p by TCS control. The view that TCS control is evolutionarily conserved is supported by several observations. (i) We demonstrated that in *S. cerevisiae*, the TEA/ATTS family transcription factor AbaAp from *A. nidulans* activates not only TCS-driven reporter genes but also *FLO11* and *PGU1* even when Ste12p is absent. In *A. nidulans*, AbaAp activates target genes by binding to AbaAp-responsive elements that are identical to TCS elements (1). As found for TCS elements in *S. cerevisiae* genes, multiple AbaAp-responsive elements are distributed predominantly in an individual arrangement in the promoter region of known target genes of AbaAp, such as *brlA*, *wetA*, *yA*, *rodA*, and *abaA* itself (1). Moreover, AbaAp activates target genes and conidiophore development independently of SteAp, a protein with similarity to Ste12p, which regulates sexual but not AbaAp-dependent asexual development (50). In this context, it should be noted that *A. nidulans* asexual spore formation involves a process that is reminiscent of yeast pseudohyphal development (14). (ii) In the human pathogen *C. albicans*, the TEA/ATTS transcription factor CaTec1p regulates target genes, serum-induced hyphal growth, and virulence primarily by a mechanism that does not require the Ste12p-like protein CaCph1p (25, 26, 47). (iii) In mammals, members of the transcriptional enhancer factor (TEF-1) family, which have the TEA/ATTS DNA-binding domain in common, activate target genes by binding to the consensus sequence GGAATG, which matches the consensus of TCS elements in a reverse orientation (20–22).

Functions and functional domains of Tec1p. The hallmark of TEA/ATTS family transcription factors is their DNA-binding domain, which consists of three putative α helices that, in the case of human TEF-1, have been demonstrated to be required for DNA binding (5, 20, 21). DNA binding and transcriptional activation have been proposed to involve either homodimer formation, as found in *A. nidulans* AbaAp (1), or heterodimer formation together with a second transcription factor, e.g., Ste12p, as shown for *S. cerevisiae* Tec1p (31), or serum response factor (SRF), as found in the case of human TEF-1 (17). Here, we have performed a functional analysis of *S. cerevisiae* Tec1p. Our results suggest that TEA/ATTS family transcription factors are able to regulate gene expression by at least two separate modes of action that involve distinct functional domains of the proteins. In the case of Tec1p, we found that gene expression is activated either by FRE control or by TCS control and that these two mechanisms are separable with respect to the structure of the Tec1p protein. We have shown that the N-terminal 257 amino acids of Tec1p, containing the conserved TEA/ATTS DNA-binding domain, are sufficient to confer cooperative FRE control together with Ste12p. This finding suggests that the N-terminal part of Tec1p might contain an Ste12p-interacting domain (Fig. 8). Although this assumption remains to be tested biochemically, it is supported by the observation that *A. nidulans* AbaAp activates FRE sites in *S. cerevisiae* in a cooperative manner that depends on Ste12p (Fig. 5). Because AbaAp and Tec1p do not share any regions showing significant similarity, other than the TEA/ATTS DNA-binding domain, cooperativity might be conferred by this

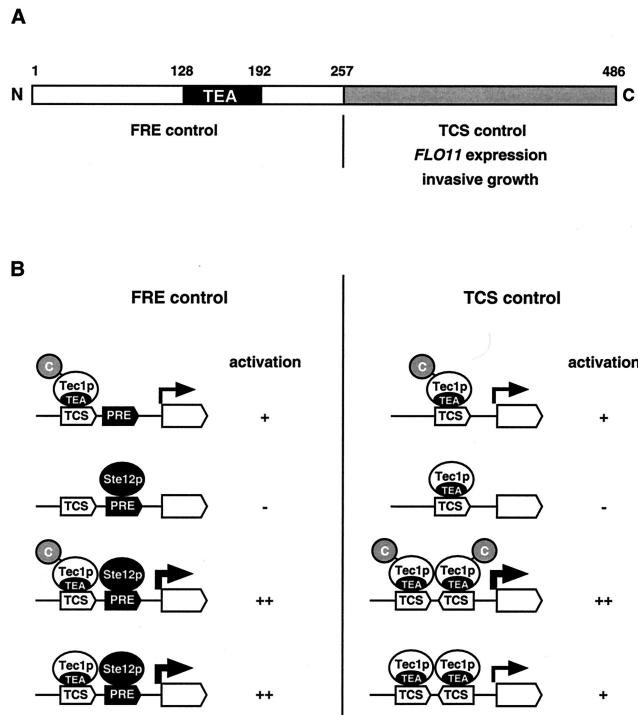


FIG. 8. Domains and functions of yeast Tec1p. (A) Domain structure of Tec1p. The N-terminal part of Tec1p encompassing amino acids 1 to 257 includes the conserved TEA DNA-binding domain and is sufficient for activation of FRE-dependent gene expression together with Ste12p (FRE control). The C-terminal domain spanning amino acids 258 to 486 is required for the activation of gene expression via TCS elements (TCS control). (B) FRE and TCS control mechanisms. High-level activation of gene expression (++) via combined TCS and PRE elements (FRE control) depends on cooperative binding of Tec1p and Ste12p and does not require the C-terminal part of Tec1p. The presence of Ste12p is not sufficient for activation (-). In contrast, the presence of Tec1p alone is sufficient for significant activation (+) of FRE-driven expression via the single TCS site. TCS control depends on the number of TCS elements and the C-terminal part of Tec1p. Single TCS elements mediate significant activation (+) of gene expression by Tec1p depending on its C-terminal part. High-level activation (++) comparable to FRE-mediated expression is achieved by combination of multiple TCS elements but also depends on the C terminus of Tec1p.

conserved part of AbaAp. A previous study has shown that the N-terminal TEA/ATTS DNA-binding domain of mammalian TEF-1 is sufficient for physical interaction with serum response factor, a MADS box family transcription factor that confers combinatorial control together with TEF-1 in gene activation (17). Therefore, one might speculate that the evolutionarily conserved TEA/ATTS domain governs not only the DNA-binding activity but also the domain for heterodimer formation with binding partners that confer combinatorial control and cooperative activation of gene expression.

Tec1p possesses an additional domain in the C-terminal part that is required for gene activation by a second mechanism, TCS control. What is the mechanism by which Tec1p executes this function? An important observation is that TCS control does not appear to involve cooperativity. Our study shows that multiple TCS elements mediate activation in a predominantly additive manner because no significant cooperative effects can

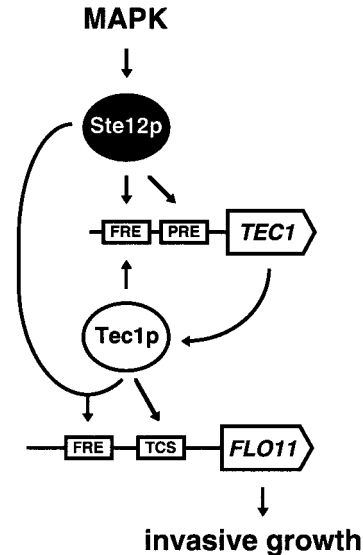


FIG. 9. Model for regulation of *FLO11* expression and invasive growth by Tec1p. The filamentation/invasion mitogen-activated protein kinase (MAPK) cascade controls Ste12p, which activates *TEC1* expression either without Tec1p by binding to PRE sites or in combination with Tec1p by binding to a single FRE site present in the *TEC1* promoter. Tec1p can activate expression of *FLO11* and invasive growth either in combination with Ste12p via the single FRE site or without Ste12p via the TCS sites present in the *FLO11* promoter.

be observed in the case of double or triple TCSs (Fig. 5). A slight cooperative effect is observed when two inverted TCS elements are separated by 11 bp. However, cooperativity is not very pronounced (1.4-fold) and not comparable to that observed for an FRE site when Tec1p acts together with Ste12p (19-fold). Remarkably, Tec1p can also activate an FRE-driven reporter gene in the absence of Ste12p. Obviously, Tec1p can bind to the TCS element within the FRE and activate transcription without Ste12p. In this case, however, cooperativity is lost and TCS control depends on the dosage of the Tec1p protein, much like that observed for TCS-driven reporters. Whether Tec1p binds to single TCS elements as a monomer or as a homodimer cannot be concluded from our study. However, biochemical studies with AbaAp suggest that it binds to single TCS elements predominantly as a monomer and not as a homodimer (1). Taken together, these findings support a mechanism by which Tec1p binds as a monomer to single TCS elements and activates transcription by involvement of its C-terminal part (Fig. 8).

What is the function of the C-terminal part of Tec1p, and why is it required for TCS control but not for FRE control? A simple answer to this questions is that the C-terminal portion of Tec1p provides the same function(s) for the TCS control mechanism as does Ste12p for the FRE control mechanism. These functions might include nuclear transport, regulation of DNA-binding activity, or interaction with the transcriptional apparatus. Whether the C-terminal part of Tec1p fulfills these functions on its own or by interaction with further partners remains to be elucidated. Interestingly, Tec1p has been found to be physically associated not only with Ste12p but also with the mitogen-activated protein kinase Kss1p (19). This opens

the possibility that FRE control and TCS control involve association of Tec1p with Kss1p. In the case of FRE control, this interaction might be established by the TEA/ATTS domain and involve Ste12p, whereas in the case of TCS control, the C-terminal part of Tec1p could associate with Kss1p independently of Ste12p. However, Ste12p might also be involved in TCS control mediated by the C-terminal part because TCS-driven expression was found to be two- to threefold less efficient in the absence of Ste12p. In this scenario, non-DNA-bound Ste12p would contribute to TCS control by interaction with the C terminus of Tec1p in a manner similar to interaction of Ste12p with the alpha-1 protein at alpha-specific genes (54).

In conclusion, our study shows that the TEA/ATTS transcription factor Tec1p fulfills more than one function in the regulation of gene expression and cellular development. These functions are executed by distinct domains of Tec1p and require combination with additional factors, e.g., Ste12p or maybe Kss1p. It will be interesting to resolve the exact temporal and spatial interactions between the different domains of Tec1p and these factors in the future.

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