Differential display analysis of mutants for the transcription factor Pdr1p regulating multidrug resistance in the budding yeast

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Abstract The transcription factor Pdr1p recognizes Pdr1p/Pdr3p-response element (PDRE) to activate genes involved in multidrug resistance of the budding yeast. To identify novel targets of Pdr1p, we compared transcriptomes among the yeast cells bearing wild, disrupted and gain-of-function alleles of *PDR1* using a high-throughput fluorescent differential display PCR. Consequently, we identified 20 transcripts apparently regulated by Pdr1p, which are derived from well-known target genes as well as those that have never been described in the context of drug resistance. Intriguingly, among the latter, a previously unrecognized gene bearing a small putative open reading frame preceded by a functional PDRE was found. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: *PDR1*; Differential display; Transcriptome

1. Introduction

The budding yeast *Saccharomyces cerevisiae* serves as an excellent model for higher eukaryotic cells in various aspects of molecular cell biology. It is also the case for multiple drug resistance, one of the most serious issues in cancer chemotherapy. The studies on drug-resistance mutants of the yeast have revealed that many genes control multiple or pleiotropic drug resistance (PDR) phenomenon through the formation of the PDR network [1]. Among these genes, *PDR1* plays a key role. The product of *PDR1* is a transcription factor belonging to Cys_2His_6-type zinc-finger protein [2]. Both Pdr1p and its homolog Pdr3p [3] bind to a characteristic sequence called Pdr1p/Pdr3p-response element (PDRE), whose consensus is TCCGCGGA, and activate the transcription of genes flanking the element [4–10].

The target genes for Pdr1p/Pdr3p include those for ATP binding cassette (ABC) protein superfamily, such as *PDR5* [4,5,11], *SNQ2* [7,12,13], *YOR1* [10,14,15], *PDR10* [9,15] and *PDR15* [9,15]. The products of these genes function as efflux pumps for various drugs. Accordingly, gain-of-function mutations in *PDR1* increase the amounts of these proteins to confer PDR phenotype, whereas the cells with compromised Pdr1p activity show hypersensitivity to various drugs [1]. It is reported that *HXT9* and *HXT11*, members of the major facilitator superfamily (MFS), are also regulated by *PDR1*/*PDR3* [8]. Intriguingly, in contrast with the ABC proteins, the deletion, but not the overexpression, of these MFS genes confers drug resistance. In addition to these membrane proteins, the expression of *PDR3* is auto-regulated by Pdr1p/Pdr3p [6].

While the transcription factor Pdr1p has been well characterized and its binding consensus sequence was identified, its downstream targets have not been fully explored. We thus launched a comparative transcriptome analysis, in which the expression level of each gene is compared among the yeast cells bearing wild-type, disrupted and gain-of-function alleles of *PDR1*. The target of Pdr1p would be down-regulated in *pdr1*Δ strains and/or induced in cells with enhanced Pdr1p activity provided by gain-of-function alleles. We thus search such transcripts using a high-throughput fluorescent differential display (FDD) PCR.

2. Materials and methods

2.1. FDD analysis

The three yeast strains used in the transcriptome analysis were FY1679-28C (*MAT***a**, *PDR1, PDR3, ura3-52, leu2Δ1, trp1Δ63, his3Δ200*), FY1679-28C/TDEC (*MAT***a**, *pdr1Δ2::TRP1, pdr3Δ::HIS3, ura3-52, leu2Δ1, trp1Δ63, his3Δ200*) and EC61 (*MAT***a**, *PDR1-3, pdr3Δ::HIS3, ura3-52, leu2Δ1, trp1Δ63, his3Δ200*), each bearing a wild-type, a null and a gain-of-function allele of *PDR1* [16]. Following the growth in YPAD (1% yeast extract, 2% peptone, 0.004% adenine sulfate, 2% glucose) at 30°C for 16 h, each strain was diluted with YPAD to $OD_{600nm} = 0.5$, cultured for an additional 2 h at 30°C and harvested for RNA isolation. Total RNAs were isolated from each strain using the modified hot-phenol method [17]. The extracted RNAs were treated with RNase-free DNase I to remove residual contaminating DNAs and further purified by acid-guanidine thiocyanate-phenol-chloroform method using TRIzol LS reagent (Gibco BRL).

FDD analysis was performed using the protocol S described previously [18–20]. Differentially displayed cDNA bands were excised from the gel, re-amplified and subjected to direct cycle sequencing with the fluorescent anchor primers. The cDNA fragments refractory to direct sequencing were cloned into a 'T-vector' and analyzed as described previously [18–20]. The expression level of each candidate target was compared among the strains using a modified adapter-tagged competitive PCR technique [21]. The promoter regions of the candidate targets were analyzed by YEBISU to search conserved sequence motifs [22].

2.2. Characterization of YPR036W-A

To reveal the structure of *YPR036W-A* transcript, 5′-RACE (rapid amplification of cDNA ends) and 3′-RACE were performed as described previously [23]. For β-galactosidase assays, we fused a 734-bp fragment, which spans the promoter and the first 7 codons, with the *lacZ* gene on a newly constructed vector pRS41K, a pRS416-deriva-

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tive bearing *KanMX* instead of *URA3*, to obtain pRS41K-YPR036W-A-lacZ. We replaced the PDRE-like sequence (TCCGCGGA) of this plasmid with an *Nco*I site (CCATGG) to obtain pRS41K-YPR036W-A-PDREΔ-lacZ. Using the pop-in–pop-out technique described previously [24], we replaced the PDRE-like sequence of FY1679-28C with an *Nco*I site, thereby generating FY1679-28Cd. The *PDR1* and *PDR3* of FY1679-28Cd were replaced with *pdr1Δ2::TRP1* and *pdr3Δ::HIS3* alleles, both amplified from FY1679-28C, to obtain FY1679-28Cd/TDEC. We used the gap-repair cloning technique to recover *PDR1* and *PDR1-3*, with their upstream (1200 bp) and downstream (600 bp) sequences, from FY1679-28C and EC61, respectively, to pRS41K for episomal expression of these genes.

3. Results and discussion

3.1. FDD analysis of pdr1 mutants

To identify potential target genes for the transcription factor Pdr1p, we intended to compare transcripts among three yeast strains, namely FY1679-28C (a wild-type strain for *PDR1*), FY1679-28C/TDEC (a *pdr1*Δ strain) and EC61 (a strain bearing a gain-of-function allele *PDR1-3*) [16]. These strains with differential Pdr1p activities were cultured in a rich medium, and total RNAs were isolated at the late logarithmic growth phase. The purified total RNAs were analyzed by FDD (Fig. 1), which we had established as a highly reliable message display PCR method with unsurpassed speed and operational safety [18–20]. In this analysis, we tested 174 primer combinations (i.e. three anchor primers × 58 arbitrary primers), which should cover ~90% of the 6000 yeast genes if we assume that all the transcripts can be detected by FDD and that the sampling by arbitrary primers is completely random [19]. We performed two independent FDD analyses using different batches of RNAs, each of which imaged ~18 000 cDNA bands. Of these, 80 fragments were reproducibly displayed more intensely in the strain with enhanced Pdr1p activity and/or more weakly in the *pdr1*Δ strain than in the other two.

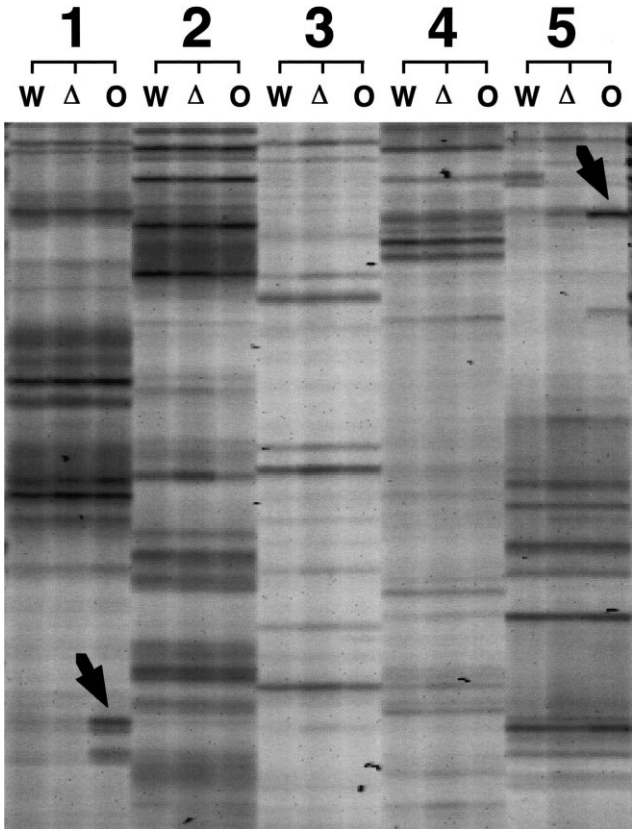

Fig. 1. A typical FDD pattern is shown, which compared transcripts from three yeast strains with differential Pdr1p activity (W, wild *PDR1*; Δ, *pdr1*Δ; O, *PDR1-3*) using five different primer combinations. The arrows indicate cDNA bands induced in the strain with enhanced Pdr1p activity (O).

3.2. Confirmation of differential expression

We succeeded in revealing the identities of 72 out of the 80 bands described above. These sequences contained those apparently derived from different portions of the same transcript: removal of such redundancy left 23 independent messages. We then confirmed their expression pattern by adapter-

Table 1
Induction of potential target genes of Pdr1p

ORF name	Gene name	Expression level		
		PDR1 (FY1679-28C)	*pdr1*Δ (FY1679C-28C/TDEC)	*PDR1-3* (EC61)
YAL066W		1.0	0.4	1.0
YBR145W	ADH5	1.0	0.5	1.9
YBR256C	RIB5	1.0	0.8	1.6
YCL030C	HIS4	1.0	1.3	3.1
YDL020C	RPN4	1.0	0.5	2.7
YDR011W	SNQ2	1.0	0.6	7.8
YGL209W	MIG2	1.0	0.9	3.4
YGL224C	SDT1	1.0	0.8	1.7
YGR254W	ENO1	1.0	0.8	2.1
YGR281W	YOR1	1.0	0.6	17.8
YHR071W	PCL5	1.0	0.6	1.5
YHR162W		1.0	0.7	1.7
YLR044C	PDC1	1.0	0.9	1.7
YMR062C	ECM40	1.0	1.4	2.3
YMR102C		1.0	0.4	1.9
YNL231C	PDR16	1.0	0.8	6.4
YOR153W	PDR5	1.0	0.0	10.0
YOR303W	CPA1	1.0	0.7	2.1
YPR036W-A		1.0	0.9	4.3
YPR145W	ASN1	1.0	0.7	1.9

Relative expression ratio for each gene was quantified using an adopter-tagged competitive PCR method among the three strains. The expression level in the wild-type cell is taken as 1.

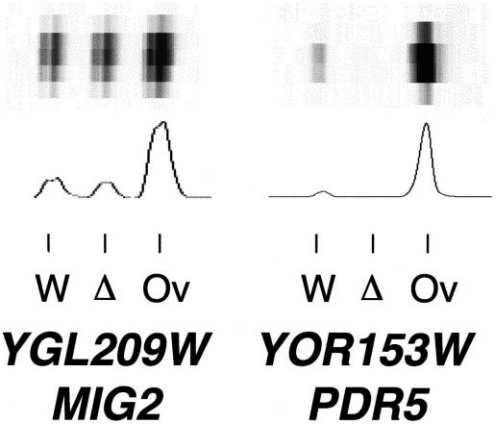

Fig. 2. Quantification of target gene induction by ATAC-PCR. The expression patterns of targets revealed by the FDD analysis were examined by a modified adapter-tagged competitive PCR [21]. Two examples, one for the established target *PDR5* and the other for a novel target *MIG2*, are shown. The upper and lower panels show gel patterns and electropherogram, respectively. Each PCR band is derived from the indicated strain (W, wild *PDR1*; Δ, *pdr1*Δ; Ov, *PDR1-3*), and their ratios quantified by GeneScan analysis software are summarized in Table 1.

tagged competitive PCR [21], a highly sensitive method to quantify the ratio of expression levels, using gene-specific primers derived from open reading frames (ORFs) lying upstream of the identified cDNA fragments (Fig. 2, Table 1). In 19 out of the 23 cases, the expression of each upstream ORF showed good correlation with the FDD pattern, thereby demonstrating the reliability of our approach.

3.3. Candidates for targets of Pdr1p

The 19 genes identified as above include *PDR5*, *SNQ2* and *YOR1* (Table 1). These three are the most well-established targets of *PDR1* encoding members of ABC superfamily. Identification of these major targets as well as a recently identified one, *PDR16* [25], proved the principle of our approach. On the other hand, we failed to detect other target genes, namely *HXT9*, *HXT11*, *PDR10* and *PDR15* [8,9]. Retrospective analysis of our failure in detecting these genes suggested that our arbitrary primers would not hit them at all or could hit, but at a position too distant from the 3′-ends to be amplified by PCR (not shown). These results demonstrated the limitation inherent to the sampling by arbitrarily selected primers. The genes other than these four are those that have never been described in the context of drug resistance. Their biochemical characters are so divergent that it is difficult to draw a simplistic explanation for their induction by Pdr1p.

To identify potential regulatory signals, we analyzed the upstream 600-bp regions of the identified targets using YEBISU program [22]. To our interest, the candidate regulatory element predicted with the highest score was exactly matched to the PDRE (Tables 2A,B). In addition to the four known targets mentioned above, *RIB5, HIS4, RPN4, MIG2, CPA1* and *ASN1* were found to contain PDRE or its variants. We also noticed that *YMR102C* has a PDRE at the positions −607 to −598. The other genes, that lack apparent PDREs, would not be the primary targets for Pdr1p, but are induced indirectly. However, to discriminate between primary and indirect targets, much finer expression analyses using conditional mutants and/or genome-wide chromatin immunoprecipitation analyses [26] will be necessary.

During the course of this work, DeRisi et al. [27] reported DNA microarray analysis of FY1679-28C/TDEC cells with or without episomal *PDR1-3* [27]. The genes activated by *PDR1-3* in their study do not include all of the targets that we identified. In addition, our microarray analysis revealed that some of the targets identified in their report fail to show higher expression in EC61 than in others. Notably, most targets identified in both studies are those bearing typical PDREs, whereas those lacking the element are barely shared between the two (M. Onda et al., in preparation). Similarly, recent microarray experiments [28] comparing the presence and absence of *PDR1-3* observed no significant induction for eight of the previously suggested targets, seven of which bear no PDREs. Intriguingly, this study revealed eight novel candidates for Pdr1p-targets including *RPN4* and *YMR102C* [28], both of which were identified also in our FDD analysis and associated with PDREs (Table 1).

Inconsistency among the results of these studies may be partly due to the difference in strain and culture conditions; we did find that some of the identified targets are expressed much more abundantly in synthetic media and hence the in-

Table 2A
PDRE-like elements extracted by YEBISU

ORF name	Gene name	Position/sequence			Score
YBR256C	RIB5	−591	AACCACGGAG	−582	9.68
YBR256C	RIB5	−124	TTCCATGGAG	−115	13.85
YCL030C	HIS4	−413	AGCCGTGGAA	−404	13.07
YDL020C	RPN4	−443	TTCCGTGGAA	−434	15.95
YDL020C	RPN4	−400	TTCCACGGAT	−391	12.55
YDR011W	SNQ2	−585	TTCCGCGGAT	−576	13.78
YGL209W	MIG2	−328	ATCCGCGGAG	−319	14.49
YGL209W	MIG2	−316	ATCCACGGAG	−307	13.26
YGR281W	YOR1	−402	TTCCGTGGAA	−393	15.95
YGR281W	YOR1	−349	ACCCGTGGAA	−340	13.07
YNL231C	PDR16	−494	AGCCACGGAA	−485	12.14
YNL231C	PDR16	−423	TTCCGCGGAG	−414	15.36
YOR153W	PDR5	−564	GTCCGTGGAG	−555	11.61
YOR153W	PDR5	−535	TCCCACGGAA	−526	13.01
YOR153W	PDR5	−492	TTCCGCGGAA	−483	16.23
YOR153W	PDR5	−376	TTCCGTGGAA	−367	15.95
YOR153W	PDR5	−314	CTCCGCGGAA	−305	12.78
YOR303W	CPA1	−501	TCCCGTGGAA	−492	13.95
YPR145W	ASN1	−332	TGCCGTGGAC	−323	10.49

Table 2B
Consensus sequence for the PDRE-like element extracted by YEBISU

	Position									
	1	2	3	4	5	6	7	8	9	10
A	32	5	0	0	30	0	0	0	100	55
C	5	16	100	100	0	55	0	0	0	5
G	5	16	0	0	70	0	100	100	0	30
T	58	63	0	0	0	45	0	0	0	10
Consensus	T	T	C	C	G	C	G	G	A	A

duction effect of *PDR1-3* is apparently blurred (unpublished observation). One may thus efficiently identify bona fide targets by searching genes similarly induced regardless of experimental conditions.

3.4. A novel tiny gene YPR036W-A induced by Pdr1p

As mentioned above, ATAC-PCR assays using ORF primers failed to confirm differential expression patterns for four cDNA bands. However, for two of the four, RT-PCR assays using primers designed within the cDNA bands reproduced the induced expression patterns in the strain with enhanced Pdr1p activity (Fig. 4B). Both of these fragments were derived from the intergenic region between *VMA13/YPR036W* and *ERV2/YPR037C* (Fig. 4A), and RT-PCR assays revealed that they are derived from a single transcript (not shown). We thus hypothesized a hitherto overlooked transcription unit lying in this region.

To know the direction of this transcript, we performed strand-specific RT-PCR assays, in which the cDNA is synthesized with one of the specific primers and subsequently used as the template for PCR with both primers. If the oligonucleotide used in the reverse-transcription step is of anti-sense direction as to the transcript, it would prime the first-strand synthesis, and hence RT-PCR product would be obtained. In contrast, if it is of the sense orientation, nothing would be amplified. The results of such strand-specific RT-PCR assays clearly indicated that the RNA is transcribed in the direction from *VMA13/YPR036W* to *ERV2/YPR037C* (Fig. 4C). We next performed 5′- and 3′-RACE to reveal the entire structure of this transcript. Since the predicted transcript does not have any ORF longer than 100 amino acids, it had not been identified as a gene in the conventional annotation of the budding yeast genome sequence. However, it has an ORF composed of 68 residues and we designated this gene as *YPR036W-A*.

3.5. PDRE regulating the expression of YPR036W-A

Intriguingly, *YPR036W-A* has a consensus PDRE sequence

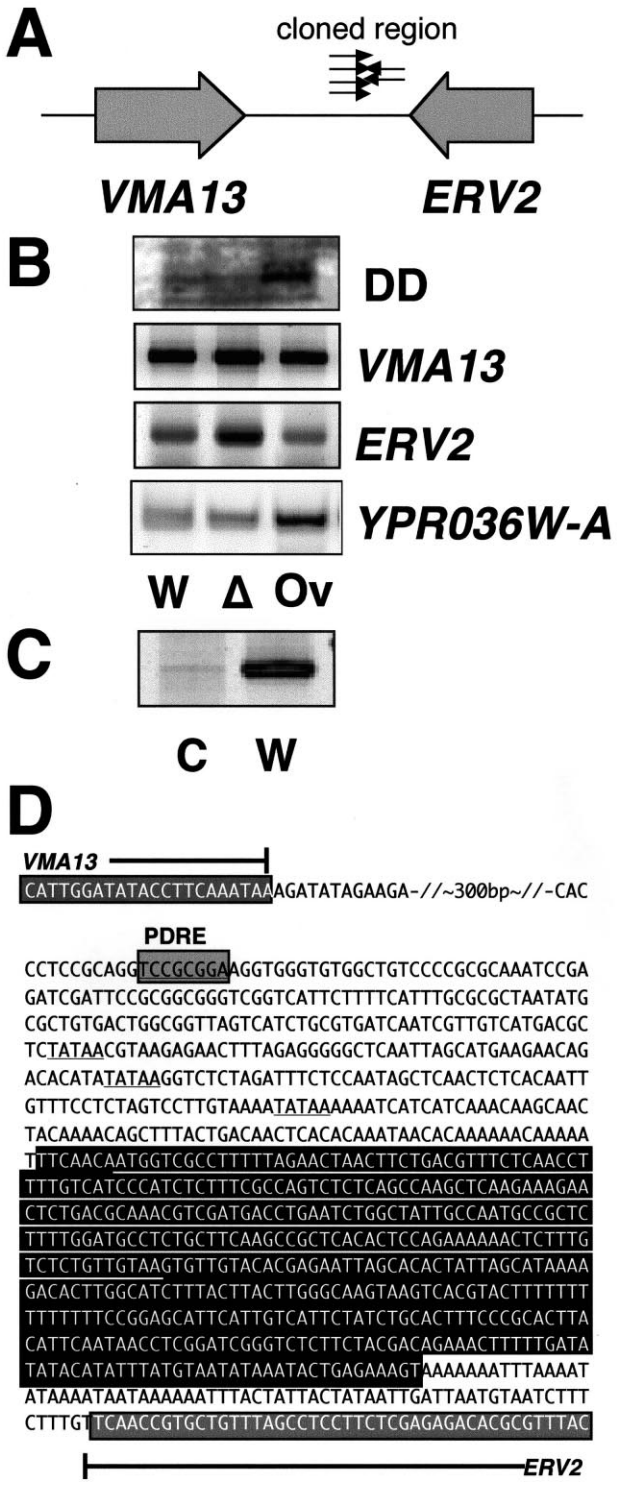

Fig. 3. A novel transcription unit lying between *VMA13* and *ERV2*. A: Six FDD fragments mapped to the intergenic region between *VMA13/YPR036W* and *ERV2/YPR037C*. B: Comparison among the FDD pattern and RT-PCR patterns for *VMA13/YPR036W*, *ERV2/YOR037C*, and the cloned FDD fragment (*YPR036W-A*). C: Strand-specific RT-PCR assay for *YPR036W-A*. Each strand-specific cDNA was synthesized by RT with each anti-sense primer, and subjected to PCR. The expected product was obtained only when cDNA for the Watson strand was used as template for PDR. D: Structure of *YPR036W-A*. The transcribed region is shaded in black, and the small putative ORF is underlined. The potential PDRE are shaded in gray, and putative TATA-like sequences are underlined. The flanking ORFs are also indicated.

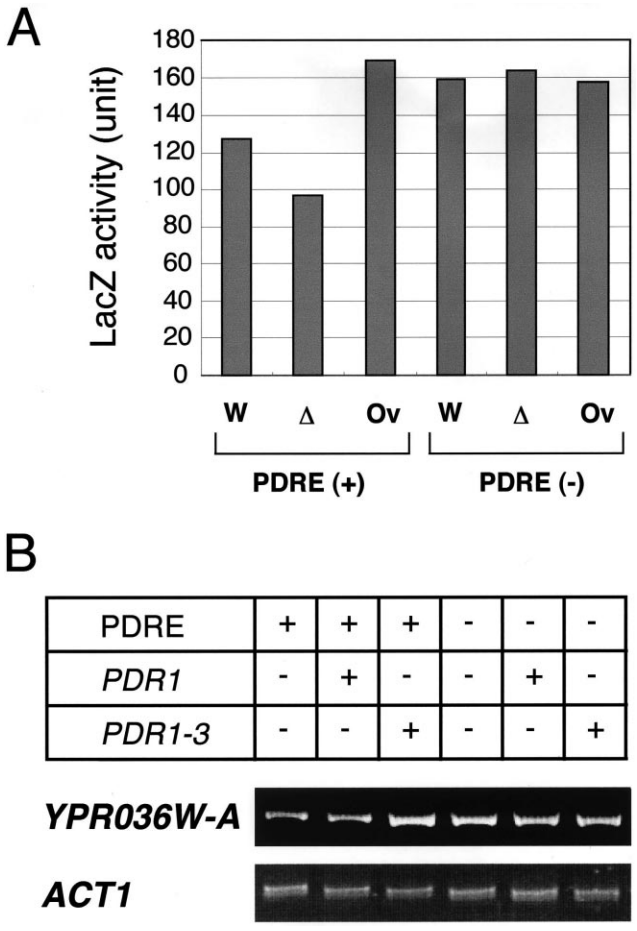

Fig. 4. Regulated expression of *YPR036W-A* by PDRE and Pdr1p. A: Using two *lacZ* reporter constructs (pRS41K-YPR036W-A-lacZ and pRS41K-YPR036W-A-PDREΔ-lacZ), activity of *YPR036W-A* promoter with or without the PDRE-like sequence was measured in strains bearing *PDR1*, *pdr1*Δ and *PDR1-3*. B: The expression of *YPR036W-A* was examined in FY1679-28C/TDEC (*pdr1*Δ, *pdr3*Δ), which bears the PDRE, and in FY1679-28Cd/TDEC (*pdr1*Δ, *pdr3*Δ, *YPR036W-A-PDRE*Δ), which lacks the PDRE, each bearing control vector, *PDR1* or *PDR1-3* plasmid. The top and bottom panels show the results of RT-PCR for *YPR036W-A* and *PDC1* (control), respectively.

(TCCGCGGA) at its −341 position (Fig. 3D). We thus tested whether or not this element regulates the expression of this gene. At first, we constructed *lacZ* reporter plasmid constructs bearing *YPR036W-A* promoter with or without the PDRE-like sequence, and introduced them into the three strains with differential Pdr1p-activities used in the FDD analysis. The construct bearing the PDRE-like sequence conferred higher *lacZ* activity in the *PDR1-3* strain than in others (Fig. 4A). In contrast, when transformed with the construct lacking the sequence, the *PDR1-3* strain failed to display higher *lacZ* activity than other strains (Fig. 4A).

Next, we intended to examine whether this sequence functions as a PDRE in the context of natural genome sequence. For this purpose, we expressed null allele (control vector), *PDR1*, or *PDR1-3* from a low-copy plasmid in *pdr1*Δ strains bearing or lacking the PDRE-like sequence, and compared the expression patterns of *YPR036W-A*. As shown in Fig. 4B, the expression level of *YPR036W-A* was well correlated with Pdr1p activity in the strain bearing the intact PDRE-like sequence, whereas such dependence was not observed at all in the strain lacking the element (Fig. 4B).

Taken together, these results indicate that the expression of *YPR036W-A* is regulated by the upstream PDRE-like sequence: the sequence does function as a PDRE. While the predicted product of this ORF shows marginal homologies to hypothetical proteins in fission yeast and nematodes (not shown), it remains elusive whether this ORF indeed encodes a protein related to multidrug resistance. Efforts to pursue this possibility are currently under way.

3.6. Conclusions

Differential display analysis of *PDR1* mutants revealed candidates for novel target genes of this transcription factor, which include a previously unrecognized transcription unit bearing a small putative ORF with a functional upstream PDRE. While message display PCR and serial analyses of gene expression are slower and less comprehensive than microarray in analyzing transcriptome, they can notably reveal novel small genes that were overlooked in the standard annotation of the genome sequence [29]. Identification of such genes is a prerequisite for truly comprehensive transcriptome analysis, and hence, for deciphering the yeast genome.

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