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RNAseq analysis of mutants in coding and non-coding transcription of phosphate genes in the yeast *Saccharomyces cerevisiae*

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ABSTRACT

In the yeast *Saccharomyces cerevisiae* phosphate starvation induces the expression of *PHO* genes, including *PHO84*, encoding an high-affinity phosphate transporter, and *SPL2*, encoding a regulatory protein. *PHO84* is down-regulated by antisense transcription. Here, using strand-specific RNAseq the effect is studied of mutations related to sense and antisense transcription of phosphate genes. Replacement of the transcriptional terminator of *PHO84* by that of *CYC1* resulted, unexpectedly, in an increased antisense transcription and a strongly reduced sense transcription of *PHO84* and a strongly reduced *SPL2* expression. The expression of unrelated genes was altered as well. The data suggest that antisense transcription of *PHO84* and not the Pho84 transporter affects the expression of *SPL2*. Deletion of the two putative binding sites for Ume6 in the *SPL2* promoter or deletion of *UME6* differently affected *SPL2* expression, suggesting that Ume6 regulates *SPL2* by a mechanism different from a simple binding to the putative Ume6 binding sites.

1. Introduction

In the yeast *Saccharomyces cerevisiae* phosphate uptake is a well-regulated process [1–5]. When phosphate is readily available, two low affinity phosphate transporters, i.e. Pho87 and Pho90, are responsible for phosphate uptake [1,6]. During phosphate starvation, the Pho4 transcription factor becomes dephosphorylated and enters the nucleus [7,8]. Here it binds to its binding sites and together with Pho2 it activates genes for adaptation to the low phosphate conditions. Activated genes include *PHO84*, encoding a high-affinity phosphate transporter [9], and *SPL2*, encoding a protein with similarity to cyclin-dependent kinase inhibitors [10]. Spl2 may downregulate low-affinity phosphate transport during phosphate limitation by targeting Pho87p to the vacuole [11]. Another well-studied *PHO* gene is *PHO5*, encoding an extracellular acid phosphatase [12].

Non-coding transcription plays an important role in regulation of gene expression in *S. cerevisiae,* including the expression of genes related to phosphate metabolism [13–16]. An example is transcription of *PHO84* in the antisense direction. Antisense transcription results in histone modifications and inaccessibility of the promoter for transcription in the sense direction and repression of *PHO84*. A similar mechanism has been proposed [16] for the regulation of *PHO5* by antisense transcription, although non-coding transcription may also be needed for activation of *PHO5* [17]. In *Schizosaccharomyces pombe* genes related to phosphate uptake are regulated by non-coding transcription as well [18–20].

In our previous study we showed that *SPL2* is expressed in approximately 90% of the cells, and is not expressed in the remaining cells [21,22]. Deletion of *RRP6*, encoding an exonuclease degrading non-coding RNA, or *BMH1*, encoding the major 14–3-3 isoform, resulted in a lower percentage of cells expressing *SPL2* and in increased transcription of sequences upstream of the *SPL2* coding region. Upon deletion of the region responsible for upstream *SPL2* transcription almost all cells express *SPL2*, indicating that the cell-to-cell variation in *SPL2* expression is dependent on the *SPL2* transcriptional start site.

In a recent paper [23], using a non-coding RNA yeast deletion collection it was shown that many deletions have a *trans* acting effect on the expression of unrelated genes often associated with effects on growth properties. Similar effects were found in fission yeast [24]. Although regulation of yeast phosphate gene expression by non-coding transcription is well documented, little is known of the effect of non-coding transcription of phosphate genes on the expression of other phosphate genes and on genome-wide gene expression. Here, the role of alternative transcription initiation of *SPL2* and of non-coding transcription of *PHO84* is further investigated by analyses of the effect of relevant mutations on genome-wide transcription.

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2. Materials and methods

2.1. Strains, primers and culture conditions

In this study the yeast strain BY4741 and strains derived from BY4741 were used, as listed in Table 1. Primers are shown in Table 2. Yeast strains were cultivated as described before [22]. Briefly, phosphate-free YNB medium (Formedium, UK) was used, supplemented with potassium phosphate (pH 5.8) to a final concentration of 7.2 mM and histidine, leucine, methionine and uracil were added to a final concentration of 20 mg/L, and potassium chloride was added to a final concentration of 50 mM. Yeast strains were grown overnight at 30 °C. This culture was used to inoculate new cultures of 50 ml of supplemented YNB yielding A_{620nm} 0.1. These cultures were incubated at 180 rpm at 30 °C and grown to A_{620nm} 0.5. For transcriptome analysis cultures were frozen immediately in liquid nitrogen.

2.2. Construction of yeast strains

For deletion of the U-box of the *SPL2* promoter by the CrisprCas technique first plasmid pML104[U(SPL2)] was constructed. To this end, plasmid pML104 [25] was digested with *Sac*I and *Swa*I and the digested vector was isolated by gel electrophoresis. Guide RNA fragment U was obtained by PCR on undigested pML104 using primers P-gRNA-5 and P-gRNA-8. The guide RNA fragment was digested with *Sac*I and ligated in pML104 digested with *Sac*I and *Swa*I. BY4741 was co-transformed [26] with 250 ng of plasmid pML104[U(SPL2)] and 1 μg of the repair fragment and transformants were selected for uracil prototrophy. The repair fragment was obtained by annealing oligo's U-repair-1 and U-repair-2. Transformants are expected to have the required deletions but they still contain the CrisprCas plasmid. The transformants were streaked on a plate containing 5-fluoro-orotic acid (1 mg/ml) and uracil in addition to methionine, histidine and leucine to select for cells that have lost the plasmid. After incubation for 5 days at 30 °C, colonies were taken and plated on plates containing or lacking uracil. Uracil auxotrophic transformants were selected and DNA was isolated. The *SPL2* promoter was analyzed by PCR using primers P-SPL2-Fw and SPL2-qPCR-4 followed by sequencing of the PCR fragments.

2.3. Transcriptome analysis by strand-specific RNAseq

RNA was isolated using an Ambion Yeast RNA kit (Life Technologies). Strand specific RNAseq, after poly A-enrichment, was performed by Genome Scan (Leiden, the Netherlands) using the Illumina NovaSeq 6000 system in the 150 bp paired end mode. At least 20 million reads were obtained for each sample. Sequence reads were analyzed using software included in the cloud based Galaxy platform (www.usegalaxxy.org) [27]. Normalization was done for each sample by calculating the number of reads per million total reads. Statistical analyses were done with EdgeR in the Galaxy platform [28]. The raw sequence data have been deposited in NCBI's Gene Expression Omnibus (GEO) and is accessible through GEO Series accession number GSE226971. All

Table 1
Yeast strains used in this study.

Strain	Genotype	Source/ Reference
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
BY4741 ΔP (GG3475)	BY4741 Δbox P	[22]
BY4741 ΔU (GG3476)	BY4741 Δbox U	This study
ume6Δ	*ume6Δ::KAN.MX* in BY4741	Euroscarf
pho4Δ	*pho4Δ::KAN.MX* in BY4741	Euroscarf
pho84Δ	*pho84Δ::KAN.MX* in BY4741	Euroscarf
BY4741 Tpho84-Tcyc1 (GG3479)	BY4741 *PHO84*(Terminator)::*CYC1* (Terminator)	[22]

Table 2
Primers.

Primer	Sequence (5'- 3')
P-gRNA-5	GGGAACAAAAGCTGGAGCTCC
P-gRNA-8	CTAGCTCTAAAACATGCTTACCGAGATTTGCCGGATCATTTATCTTTCACTGCGGAG
U-repair-1	AAAGAGAGACCGTACCGCAATAAAATGGACCCTTGTCGGTCACGTGAGCAAAAATACTATAACCGAACCATCATTGGGGTAGGAAACATTATAAATATTTGATATATATAAAGGAAAAA
U-repair-2	TTTTTCCTTTATATATCAAATATTTATAATGTTTCCTACCCCAATGATGGTTCGGTTATAGTATTTTTTGCTCACGTGACCGACAAGGGTCCATTTTATTGCGGTACGGCTCTCTCTTT
P-SPL2-Fw	AAA GAGCTC TTTACACTGGGATATTTACAAGAC C
SPL2-qPCR-4	GCTGTACCGCCAAGGTAGAT

normalized read counts are shown in Supplementary Table S1.

3. Results

In this study the regulation of phosphate gene expression was addressed in exponentially growing yeast cells in minimal medium in the presence of phosphate. We especially focused on the expression of *PHO84* and *SPL2*. An overview of some regulatory elements for the two genes is given in Fig. 1. It has well been established that upon phosphate depletion the expression of both *PHO84* and *SPL2* is strongly induced and that the transcription factor Pho4 plays a critical role. A putative binding site for this transcription factor (CACGTG) can be found in the *PHO84* promoter (orange block in Fig. 1A). It has further been shown that the expression of *PHO84* is strongly reduced by transcription in the antisense direction (indicated by a red arrow in Fig. 1A). In the *SPL2* promoter four putative Pho4 binding sites can be found (orange blocks in Fig. 1B). In addition, putative binding sites for many other transcription factors can be found in the *SPL2* promoter, including two binding sites for the transcriptional regulator Ume6 (green blocks in Fig. 1B) [29]. In our previous paper [22], we showed that the expression of *SPL2* may be regulated by alternative transcription initiation, leading to either non-coding upstream RNA or coding RNA (red and green arrows, respectively, in Fig. 1B). In order to further study the regulation of phosphate gene expression in *S. cerevisiae* several mutants were used to investigate the effect of the mutations on genome-wide gene expression. The mutants included deletion mutants of the P-box and the U-box of the *SPL2* promoter (Fig. 1B), deletion mutants of the *UME6*, *PHO4* and *PHO84* genes and a mutant in which the transcriptional terminator of *PHO84* has been replaced by the transcriptional terminator of *CYC1*. For our gene expression studies the yeast strains were grown in YNB medium supplemented with the required nutrients and containing phosphate to an A_{620nm} of 0.5. Genome-wide RNA levels were determined by RNAseq analysis. An overview of these RNA levels is given in Supplemental Table S1.

To visualize the effect of the mutations on the levels of *SPL2* and *PHO84* coding and non-coding RNA the stand-specific RNA reads were aligned to the genomic sequences of these two genes. As shown in Fig. 2A RNA reads aligned to the *SPL2* open reading frame and a small number of the reads to the upstream region (green arrow in Fig. 2A) in agreement with our previous study (for quantification, see Table 3A). Upon deletion of the P-box reads aligning to the upstream region were almost absent and the number of reads aligning to the open reading frame increased (8 and 19 reads per million for BY4741 and BY4741ΔP, respectively) in agreement with our previous study [22]. Deletion of the U-box had a smaller effect on the number of reads aligning to the upstream region, and the number of reads aligning to the open reading frame increased. The U-box contains two putative Ume6 binding sites. Deletion of the *UME6* gene lead to a decreased number of RNAseq reads aligning to the open reading frame and a small increase in the number of reads aligning to the upstream region (ume6Δ in Fig. 2A). Deletion of *PHO4* resulted in a low number of reads aligning to the upstream region and to the open reading frame of *SPL2* (pho4Δ in Fig. 2A), confirming the role of the Pho4 transcription factor in the expression of *SPL2*. Deletion of *PHO84* did not significantly affect the expression of *SPL2* (pho84Δ in Fig. 2A). Surprisingly, upon replacement of the *PHO84* transcriptional terminator region by that of *CYC1* (strain BY4741Tpho84-Tcyc1) almost no RNA reads aligned to the *SPL2* region (Tpho84 in Fig. 2A).

RNAseq reads of the control strain BY4741 aligned to *PHO84* in the sense direction (Fig. 2B, indicated in green). In addition, a minority of the reads aligned in the antisense direction, in agreement with previous observations (Fig. 2B, indicated in red). Deletion of either the P-box or U-box in the *SPL2* promoter resulted in an increased number of *PHO84* reads in the sense direction, whereas deletion of the *UME6* gene resulted in a decreased number of reads aligning in the sense direction (Fig. 2B, Table 3B). Deletion of *PHO4* resulted in the absence of reads aligning in the sense direction, and a slightly increased number of reads aligning in the antisense direction. This indicates that Pho4 is essential for the expression of *PHO84*, not only during phosphate starvation, but also under standard growth conditions. On the other hand, Pho4 is not required for the expression of *PHO84* antisense RNA. With the goal to block *PHO84* antisense transcription, the *PHO84* transcriptional terminator region was replaced by that of *CYC1*. However, the effect was completely opposite to the expected effect (Fig. 2B, Table 3B). Reads aligning in the sense direction were completely absent and reads aligning in the antisense direction were increased. Details of the position of the *CYC1* terminator sequences in the chromosomal context as well as details of the alignment of RNA reads from BY4741Tpho84-Tcyc1 and from BY4741 to these sequences are shown in Supplementary Fig. S1.

To see whether the mutations also affected the RNA levels of other genes involved in phosphate metabolism we selected nine phosphate genes (*SPL2*, *PHO84*, *PHO4*, *PHO5*, *PHO11*, *PHO12*, *PHO89*, *PHM6*, *VTC3*) and the control gene *ACT1* and visualized the effects in Fig. 3. For comparison, the effects of deletions of *BMH1* and *SPL2* studied in our previous work [21], were included. As expected, *PHO84* and *SPL2* were affected the most by the mutations. The effects of deletions of *BMH1*,

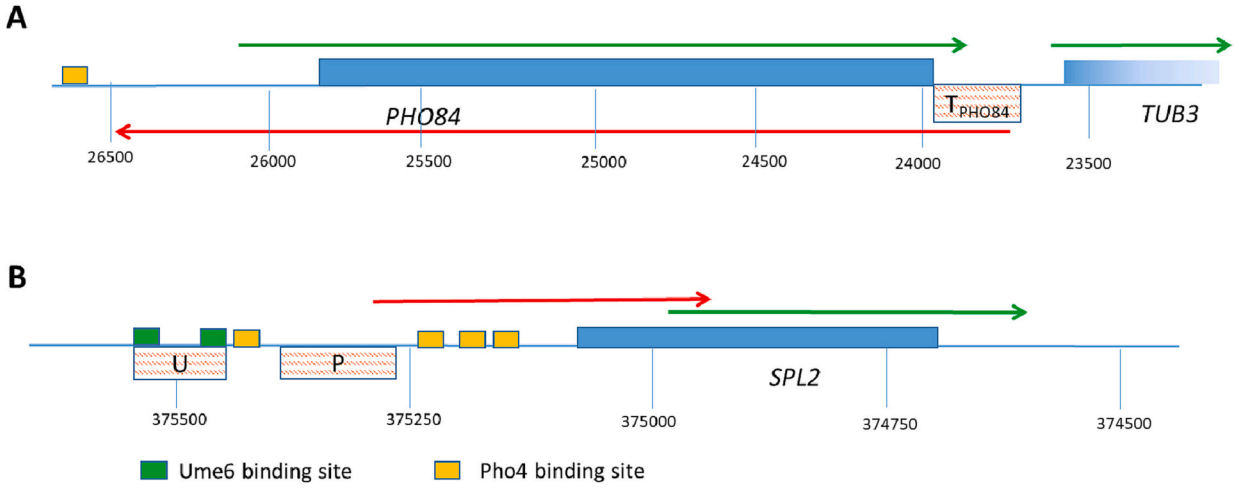

Fig. 1. Model of the transcription of the *PHO84* (A) and *SPL2* (B) genes. Green arrows, coding transcription; red arrows, non-coding transcription. Red-striped boxes, sequences deleted in this study. Coordinates are taken from SGD (www.yeastgenome.org). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

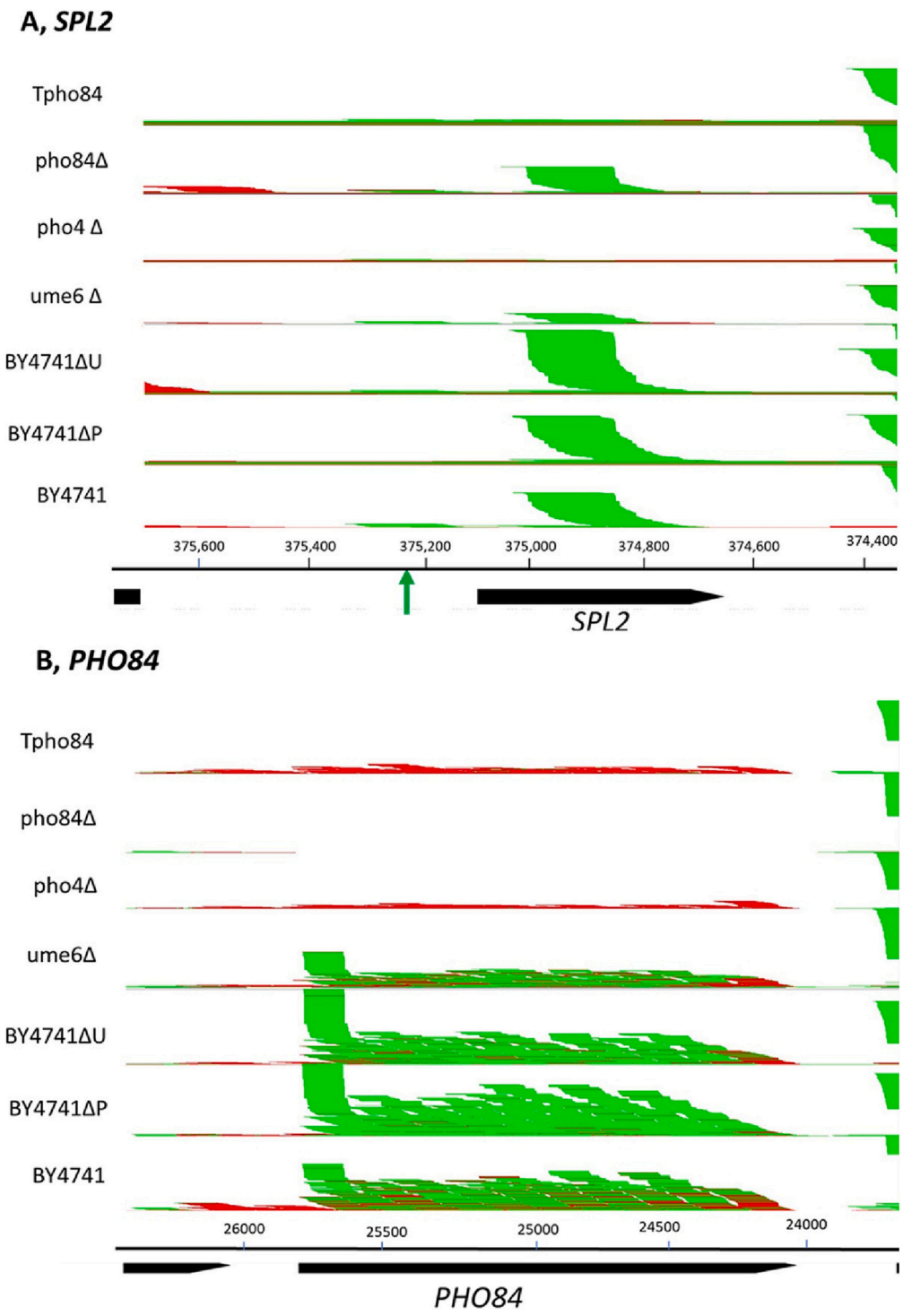

Fig. 2. Alignment of strand-specific RNA reads to the *SPL2* (A) and *PHO84* (B) genes. Reads aligning in the sense direction are indicated in green, reads aligning in the antisense direction are indicated in red. Green arrow highlights the reads corresponding to *SPL2* upstream transcription. Alignments were visualized using Integrated Genome Browser [38]. Coordinates are obtained from the Saccharomyces Genome Database (www.yeastgenome.org). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

SPL2, and replacement of the *PHO84* terminator on the phosphate genes were quite similar. On the other hand, the effect of deletion of the *PHO84* open reading frame was different. A clear positive effect of this deletion on the RNA levels of *PHO5* and *PHO89* (encoding an acid phosphatase and a plasma membrane Na^+/Pi cotransporter, respectively) was observed. Upon replacement of the *PHO84* terminator the levels of *PHO84* RNA were very low, but the expression of *PHO5* and *PHO89* was not increased.

The studied mutations not only affected phosphate metabolism, but also other processes, although to a variable extent. The number of significantly affected genes is given in Table 4, and the affected genes are shown in Supplementary Table S2. Deletion of the P-box from the *SPL2* promoter had only a minor effect whereas deletion of the U-box had much more effect. Searching for GO-terms (https://www.yeastgenome.org/goTermFinder) enriched in the set of down-regulated genes upon deletion of the U-box revealed the GO-term 'methionine metabolic process' with only three genes (*BAT1, MIS1*, *SAM2*). The wider effect of deletion of the U-box may be related to the slow growth phenotype of the U-box deletion mutant. Deletion of *UME6* resulted in upregulation of many genes, most of them related to sporulation, in agreement with the reported role of *UME6* in the sporulation process [30,31]. Deletion of *PHO4*, encoding a transcription factor involved in the response to low

Table 3
Quantification of the number of RNAseq reads aligning to *SPL2* and *PHO84*.

A. Reads aligning to *SPL2* coding sequences and upstream sequences (in reads per million; SD, standard deviation; n, number of samples; *t*-test, *P* value obtained by the t-test of the difference relative to BY4741

		Average	SD	n	T-test
BY4741	coding	8.2	3.3	5	
	upstream	0.4	0.1	5	
BYΔP	coding	19.0	3.2	3	0.004
	upstream	0.0	0.0	3	0.003
BYΔU	coding	19.4	5.2	3	0.009
	upstream	0.5	0.2	3	0.305
ume6Δ	coding	2.6	0.9	3	0.029
	upstream	0.7	0.0	3	0.009
pho4Δ	coding	0.3	0.3	2	0.023
	upstream	0.3	0.1	2	0.417
pho84Δ	coding	5.7	0.2	2	0.355
	upstream	0.4	0.2	2	0.987
Tpho84	coding	0.3	0.1	3	0.007
	upstream	0.4	0.1	3	0.556

B. Reads aligning to *PHO84* in the sense and antisense direction (in reads per million; SD, standard deviation; n, number of samples; T-test, P value obtained by the T-test of the difference relative to BY4741)

		Average	SD	n	T-test
BY4741	sense	117	27	5	
	antisense	27	3	5	
BYΔP	sense	342	56	3	0.0002
	antisense	18	1	3	0.0057
BYΔU	sense	260	86	3	0.0115
	antisense	31	8	3	0.3328
ume6Δ	sense	56	42	3	0.0450
	antisense	32	2	3	0.0477
pho4Δ	sense	0	0	2	0.0023
	antisense	36	2	2	0.0159
pho84Δ	sense	0	0	2	0.0023
	antisense	0	0	2	0.0001
Tpho84	sense	1	0	3	0.0004
	antisense	39	2	3	0.0011

phosphate, resulted in the down-regulation of a modest number of genes related to phosphate metabolism. Surprisingly, replacement of the *PHO84* terminator also affected other genes. Searching for GO-terms enriched in the set of up-regulated genes revealed the GO-terms 'glucose 6-phosphate metabolic process', 'generation of precursor metabolites and energy', 'pentose-phosphate shunt', 'NADPH regeneration, and 'NADP metabolic process', suggesting that genes involved in primary metabolism are especially affected. Most of these genes were not significantly affected by deletion of *PHO84*.

4. Discussion

In this study we addressed the role of non-coding transcription of *SPL2* and *PHO84* in a genome-wide context. To this end, the effects of mutations affecting non-coding transcription of the *PHO84* and *SPL2* genes were studied by strand-specific RNAseq analyses. With the goal to block antisense transcription of *PHO84* the transcriptional termination region was replaced by that of *CYC1* (strain BY4741Tpho84-Tcyc1) [22]. Unexpectedly this replacement resulted in an increased number of reads aligning to *PHO84* in the antisense direction and almost no reads aligning in the sense direction. This decrease may be caused by increased antisense transcription or by instability of the sense *PHO84* RNA with an altered 3′-end. In our previous study [22] we transformed strain BY4741Tpho84-Tcyc1 with plasmids containing GFP under control of the *PHO84* promoter and showed that the expression of GFP was much lower than in the control strain (13 vs. 55, arbitrary units, for BY4741Tpho84-Tcyc1 and BY4741, respectively). One possible explanation is that the increased level of antisense RNA originating from the chromosomal copy of P*HO84* has a *trans* inhibitory effect on the *PHO84* promoter on the plasmid. Evidence has been published before for *trans* effects of *PHO84* antisense RNA [32]. Other studies give evidence that *cis* effects of antisense transcription are important for *PHO84* silencing [15]. Silencing may result from antisense transcription through the promoter rather than the static accumulation of antisense RNAs at the repressed locus. Histone deposition by the HIR complex may have an important role [33].

Replacement of the *PHO84* transcriptional terminator also resulted in a strongly reduced number of RNA reads aligning to *SPL2*. This is in line with our previous study [22] in which we showed that transformation of strain BY4741Tpho84-Tcyc1 with a plasmid containing GFP under control of the *SPL2* promoter lead to a much lower expression of GFP compared to that of the corresponding control strain (1.5 vs. 3.4, arbitrary units, for BY4741Tpho84-Tcyc1 and BY4741, respectively). In contrast, in a strain in which the coding region of *PHO84* was deleted, the expression of *SPL2* was hardly affected. These observations suggest that *PHO84* antisense transcription affects *SPL2* expression and that the lower *SPL2* expression in BY4741Tpho84-Tcyc1 is not caused by a lower phosphate uptake due to the low level of the Pho84 transporter. Using fluorescent in situ hybridization it was shown that antisense *PHO84* RNA did not accumulate at the *PHO84* gene rather was exported to the cytoplasm [15]. Here it may bind proteins, e.g. transcription factors or other proteins, involved in the regulation of *SPL2* expression. Alternatively, antisense *PHO84* RNA may bind to the *SPL2* gene or it may affect the binding of transcription factors to the *SPL2* promoter. The first possibility is less likely as the homology between the antisense RNA and the *SPL2* gene is very limited. Mechanisms in which non-coding RNA acts via transcription factors have been suggested before [23,34,35]. Other mechanisms are possible as well (for review see [36]). Further research is needed to elucidate the exact mechanism of the regulation of *SPL2*, and other genes, by *PHO84* antisense transcription.

Deletion of the U-box from the *SPL2* promoter resulted in an increased level of *SPL2* coding RNA. This deletion affects not only *SPL2* but also many unrelated genes. This may be caused by the slow growth phenotype of the U-box deletion mutant. In a recent study [23] it was shown that deletion of several genes responsible for non-coding transcription also resulted in a slow-growth phenotype. In addition, these deletions greatly affected the expression of distant genes, although to a varying extent. As the U-box contains two putative Ume6 binding sites, it was interesting to investigate the effect of deletion of *UME6* on *SPL2* expression. *UME6* encodes a key transcriptional regulator of early meiotic genes and Ume6 is a Rpd3L histone deacetylase complex subunit [37]. As expected, deletion of *UME6* mainly affected genes related to sporulation and meiosis. It also resulted in a decreased number of RNAseq reads aligning to the *SPL2* open reading frame and an increase in the number of reads aligning to the *SPL2* upstream region (ume6Δ in Fig. 2A). This effect is in contrast to the effect of deletion of the U-box, suggesting that Ume6 is involved in the regulation of *SPL2* by a mechanism different from a simple binding to the putative Ume6 binding sites in the U-box. In our previous study we showed that the cell-to-cell variation of *SPL2* expression is related to the transcriptional initiation site, where transcription of the upstream region leads to cells not expressing *SPL2*, whereas transcription of the coding region leads to cells that do express *SPL2* [22].

From the data presented in this paper it can be concluded that the amount of *PHO84* antisense RNA is dependent on the termination region for *PHO84* sense transcription. Upon replacement of this termination region by that of *CYC1* the amount of *PHO84* antisense RNA is strongly increased. This replacement caused not only a strongly reduced amount of *PHO84* sense RNA but also a reduced amount of *SPL2* RNA. In addition, unrelated genes are significantly affected, six of them positively and 35 negatively. Many of these genes are related to primary metabolism, suggesting a link between phosphate uptake and metabolism. Previously [21] we found that deletion of *SPL2* did not affect primary metabolism, suggesting that the Spl2 protein is not involved in the

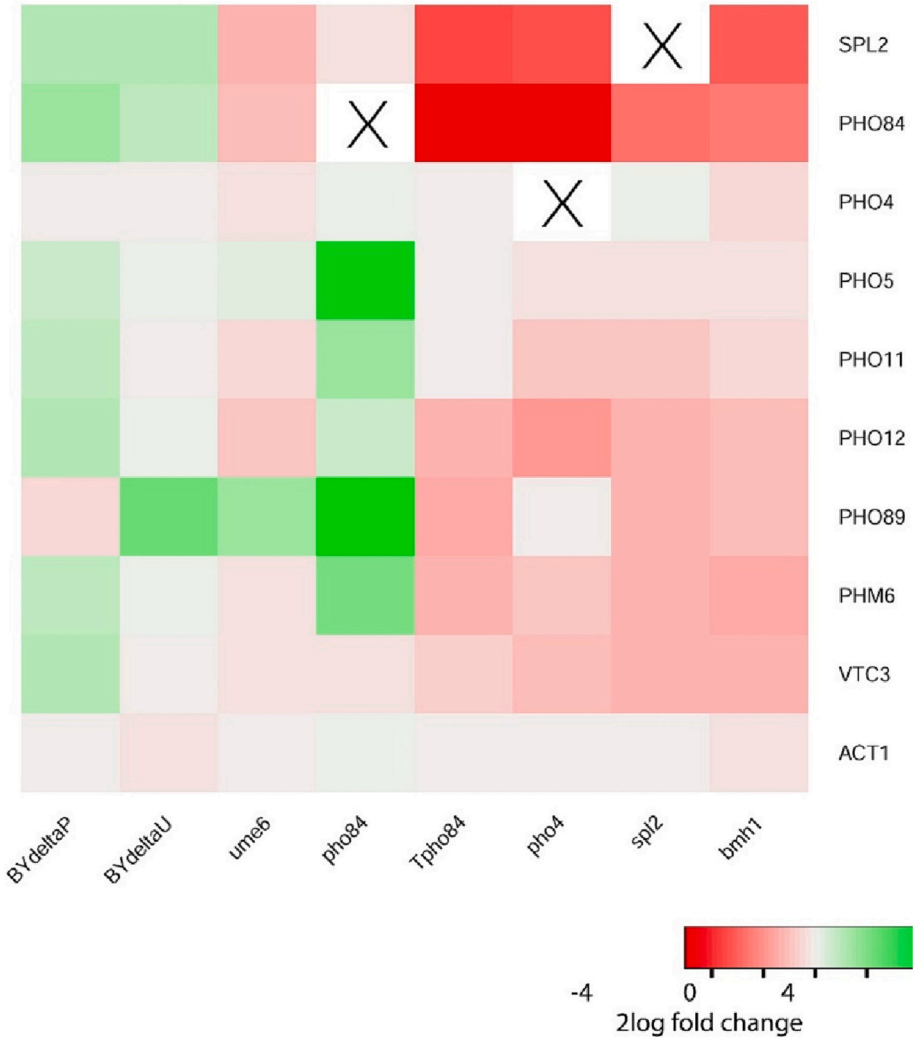

Fig. 3. Heatmap of the effect of the studied mutations on the RNA levels of selected phosphate genes and the *ACT1* control. The heatmap was constructed using the Heatmap2 program of the Galaxy platform. Upon deletion of *SPL2*, *PHO84* and *PHO4* almost no RNAseq reads aligned to the deleted genes. As this is a direct effect of the deletion, and the biological significance is absent, these data are not shown in the figure.

Table 4
Number of genes significantly affected by the studied mutations. Significance was determined by the EdgeR program in the Galaxy platform (EdgeR: log cpm > 1; FDR < 0.01; 2logFC > 1 or 2logFC < -1).

Strain	Number of up-regulated genes	Number of down-regulated genes
BY4741ΔP	0	3
BY4741ΔU	173	18
ume6Δ	358	24
pho4Δ	1	7
pho84Δ	9	1
BY4741 Tpho84-Tcyc1	6	35

regulation of primary metabolism. The mechanism by which *PHO84* antisense RNA regulates the expression of other genes is an interesting topic for future studies. The expression of *SPL2* is not only affected by *PHO84* antisense RNA, but is also dependent on *PHO4*, encoding the key transcription factor for the response to phosphate starvation. Deletion of the P-box from the *SPL2* promoter resulted in the absence of *SPL2* upstream RNA and increased levels of *SPL2* coding RNA and *PHO84* sense RNA, but hardly affected other genes. On the other hand, deletion of the U-box had a similar effect on *SPL2* RNA, but greatly affected unrelated genes, by an unknown mechanism. This may be related to the slow growth phenotype of the U-box deletion mutant. The U-box contains two putative Ume6 binding sites. As deletion of the *UME6* gene affected *SPL2* in a different way, Ume6 regulates *SPL2* via a mechanism different from a simple binding of Ume6 to its putative binding sites. As Ume6 regulates many genes related to meiosis and sporulation, Ume6 may regulate *SPL2* during the sporulation process, although this has not yet been reported.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2023.110672.

Author statement

The design of the research, the experimental work and the preparation of the manuscript were done by GPH van Heusden.

Declaration of Competing Interest

The author declares no conflict of interest.

Data availability

Data will be made available on request.

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