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Transcriptional profiling of *Neurospora crassa* Δ*mak-2* reveals that mitogen-activated protein kinase MAK-2 participates in the phosphate signaling pathway

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ABSTRACT

The filamentous fungus *Neurospora crassa* is an excellent model system for examining molecular responses to ambient signals in eukaryotic microorganisms. Inorganic phosphate (Pi) is an essential growth-limiting nutrient in nature and is crucial for the synthesis of nucleic acids and the flow of genetic information. The genetic and molecular mechanisms controlling the response to Pi starvation in *N. crassa* include at least four genes (*nuc-2, preg, pogv*, and *nuc-1*), which are involved in a hierarchical regulatory activation network. In a previous work, we identified a number of genes modulated by NUC-2 protein, including the *mak-2* gene, which codes for a mitogen-activated protein kinase (MAPK), suggesting its participation in the phosphate signaling pathway. Thus, to identify other genes involved in metabolic responses to exogenous phosphate sensing and the functioning of the MAPK MAK-2, we performed microarray experiments using a *mak-2* knockout strain (Δ*mak-2*) grown under phosphate-shortage conditions by comparing its transcription profile to that of a control strain grown in low- and high-phosphate cultures. These experiments revealed 912 unique differentially expressed genes involved in a number of physiological processes related to phosphate transport, metabolism, and regulation as well as posttranslational modification of proteins, and MAPK signaling pathways. Quantitative Real-time PCR gene expression analysis of 18 selected genes, using independent RNA samples, validated our microarray results. A high Pearson correlation between microarray and quantitative Real-time PCR data was observed. The analysis of these differentially expressed genes in the Δ*mak*-2 strain provide evidence that the *mak-2* gene participates in the hierarchical phosphate-signaling pathway in *N. crassa* in addition to its involvement in other metabolic routes such as the isoprenylation pathway, thus revealing novel aspects of the *N. crassa* phosphorus-sensing network.

1. Introduction

All living organisms sense changes in the environment and nutrient availability through a complex adaptive signaling network whose appropriate cellular responses are crucial for regulating growth, proliferation, and apoptosis. Ambient signals are detected by cellular sensors initiating the flow of intracellular information that is transmitted via transduction pathways to a target (Bahn et al., 2007; Kholodenko and Birtwistle, 2009; Richthammer et al., 2012). Numerous ambient signals stimulate mitogen-activated protein kinase (MAPK) cascades, which are conserved multi-component molecular platforms that effectively process and propagate molecular information. In fungi, a number of proteins such as transcription factors and protein kinases are phosphorylated by MAPKs, and these proteins affect gene expression, metabolism, cell division, and cell survival (Molina et al., 2010; Qi and Elion, 2005; Saito, 2010). In *Neurospora crassa*, a filamentous fungus that undergoes multicellular developmental phases, three MAPK modules have been identified, in which a series of three protein kinases phosphorylate and activate one another (Lichius et al., 2012; Maerz et al., 2008). The MAK-2 signaling pathway includes

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at least two other MAPKs: NRC1 and MEK-2, which are involved in hierarchical activation of MAK-2. Although these MAPK modules are activated by specific adaptive responses, they share common components. Genetic interactions between the kinase COT1 and the MAK-1 and MAK-2 signaling pathways are necessary for regulation of various processes, including hyphal fusion, cell wall integrity, and consequently, growth as well as sexual development in *N. crassa* (Bidard et al., 2012; Fleissner et al., 2009; Fu et al., 2011; Hamel et al., 2012; Maerz et al., 2008; Pandey et al., 2004; Park et al., 2008; Wang et al., 2012). However, the molecular functions of these conserved MAPK cascades have not been fully characterized.

Inorganic phosphate (Pi), an essential growth-limiting nutrient in nature, is a crucial component of genetic information molecules, energy transport molecules, synthesis of membrane phospholipids, and cellular signaling processes in general. Thus, maintaining the phosphate supply is important for the survival of microorganisms, which have evolved signal transduction pathways that enable them to sense the availability of Pi and modulate metabolic activities so that Pi can be acquired (Lenburg and O'Shea, 1996; Paytan and McLaughlin, 2007). Genetic and molecular mechanisms controlling the response to Pi starvation in the filamentous fungus *N. crassa* includes at least four regulatory genes (*nuc-2*, *preg*, *pgov*, and *nuc-1*), which are involved in a hierarchical regulatory activation network. Pi availability is sensed by the *nuc-2* gene, the transcription of which is regulated in response to extracellular Pi changes (Gras et al., 2009; Metzenberg, 1979; Peleg et al., 1996b). NUC-2, an ankyrin repeat protein (Li et al., 2006), transmits a metabolic signal downstream of the hierarchical regulatory pathway, inhibiting PREG-PGOV complex function under conditions of Pi shortage. This allows translocation of the transcriptional regulator NUC-1 into the nucleus (Leal et al., 2009; Peleg et al., 1996a). PREG is a cyclin-like protein kinase and PGOV is a cyclin-dependent protein kinase, whereas NUC-1 is a member of the basic helix–loop–helix (bHLH) family of transcription factors (Kang, 1993; Kang and Metzenberg, 1993; Leal et al., 2007; Robinson and Lopes, 2000). Under conditions of Pi shortage, this hierarchical regulatory network removes the repression of genes coding for nucleases, phosphatases, and Pi transporters that are necessary for fulfilling the Pi requirements in the cell (Gras et al., 2007; Leal et al., 2007; Lenburg and O'Shea, 1996; Metzenberg, 1979). Numerous genes modulated by NUC-2 protein under Pi shortage were previously identified, including the *mak-2* gene (Gras et al., 2007), suggesting that the MAPK cascade is somehow involved in the phosphate signaling pathway. Moreover, the identification of genes modulated by the *nuc*-2, *preg*, and *nuc*-1 genes revealed various aspects of the phosphorous-sensing network, including molecular events implicated in the *preg* convergent regulation of Pi, nucleotide, and energy metabolism (Gras et al., 2009; Leal et al., 2007) , stressing the significant level of complexity involved in metabolic regulation under Pi-shortage conditions in *N. crassa*.

To identify other genes involved in metabolic responses to Pi shortage and to further characterize the functioning of the *mak-2* gene in *N. crassa*, we performed microarray analysis comparing the transcription profile of a strain carrying the *mak-2* gene knockout to control, both strains grown in low-Pi cultures. Pi shortage is a nutritional condition in which the Pi-sensing network is active allowing the transcription of Pi-repressed genes in *N. crassa*, and other filamentous fungi (Caddick et al., 1986; Peleg et al., 1996a). We also compared the transcription profile of control strain grown in low- and high-Pi cultures. Here, we provide evidence that *mak-2* gene is involved in the adaptive response to extracellular Pi changes. We also report the identification of a number of genes that are down- and up-regulated in a *N. crassa* Δ*mak-2* strain that are responsive to extracellular Pi changes.

2. Materials and methods

2.1. Strains, culture conditions, and RNA extraction

Wild-type strain St.L.74-OR23-1VA (control strain) (FGSC No. 2489), and the *nuc-1A* (FGSC No.1995), *nuc-2A* (FGSC No. 1996), and Δ*mak-2* (FGSC No. 11482) mutant strains of *N. crassa* used in this study were obtained from the Fungal Genetics Stock Center, University of Missouri, Kansas City, MO (McCluskey, 2003) (www.fgsc.net). The *nuc-1* and *nuc-2* mutations were induced in the St.L 74A strain using UV light, and the mutant strains were selected for their inability to utilize RNA or DNA as a phosphorus source (Ishikawa et al., 1969; Peleg et al., 1996b). The *mak-2* knockout strain (Δ*mak*-2) was generated using the *Neurospora* Functional Genomics Project Strains (www.fgsc.net). The strains were maintained on slants of Vogel's medium (1.5% agar, 2% sucrose). When desired, the Vogel's medium was supplemented with 0.1% DNA (Deoxyribonucleic Acid Sodium Salt from Salmon Tests, Sigma Aldrich) as the sole phosphorus source.

For gene expression assays, conidia from each strain (approximately 10^6 cells mL^{-1}) were germinated for 5 h at 30 °C in an orbital shaker (200 rpm), in low- and high-Pi media (final concentrations, 10 μM or 10 mM Pi, respectively) adjusted to pH 5.4 using 50 mM sodium citrate, supplemented with 44 mM sucrose as the carbon source, and prepared as previously described (Gras et al., 2007; Nyc et al., 1966).

Total RNA was extracted from approximately 100 mg mycelia using the Illustra RNAspin mini isolation kit (GE Healthcare, Little Chalfont, UK), treated with RNAse-free DNAse I (Invitrogen, Carlsbad, CA), and purified using the RNAeasy kit (Qiagen, Hilden, Germany). RNA quality was evaluated using the Agilent Bioanalyser platform 2100 (Agilent Technologies, Santa Clara, CA).

2.2. cDNA synthesis and labeling and microarray hybridization

cDNA synthesis and labeling were performed using the ChipShot Indirect Labeling and Clean-Up System (Promega, Fitchburg, WI). The Cy3 and Cy5 cyanine dyes (GE Healthcare) were incorporated into the cDNAs by adding Cy3 or Cy5 monofunctional N-hydroxysuccinimide ester dye to the cDNA solution for 1 h at 22 °C. cDNA was subsequently cleaned using a ChipShot membrane column and dried under a vacuum according to the manufacturer's instructions.

Microarray experiments were performed using *N. crassa* array slides obtained from the Fungal Genetics Stock Center (www.fgsc.net). A detailed description of the oligonucleotide gene set is available from the *Neurospora* Functional Genomics Database (http://www.yale.edu/townsend/Links/ffdatabase/introduction.html). Washing of the pre-hybridization and hybridization slides were performed using the Pronto!™ hybridization kit (Corning Life Sciences, Corning, NY) according to the manufacturer's instructions. Scanning of the hybridized slides was performed using a Genepix 4000B scanner (Axon Instruments, Union City, CA). All hybridizations were performed in duplicate with 4 independent biological replicates, using the dye swap incorporation system. GenePix Pro6 software was used to quantify hybridization signals.

2.3. Data analyses

Microarray low-quality spots were flagged automatically by GenePix software, and each slide was subsequently inspected manually. Hybridized spots with a mean fluorescence intensity for at least one of the Cy3 and Cy5 dyes that was greater than the mean intensity of the background plus 2 standard deviations were

further analyzed. Data normalization was performed using regression technique LOWESS resulting in the $\log_2$ ratios (M values) and the average $\log_2$ intensities (A value) of Cy3 and Cy5 signals for each array (Koide et al., 2006). Differentially expressed genes were identified using Significance Analysis of Microarray (SAM) analysis with the TIGR MEV program (http://www.tigr.org/software). A false discovery rate (FDR) of 5% and a $\log_2$ ratio of 1.5, i.e., displaying at least 2.8-fold change difference in gene expression level, were set as a threshold. Statistical analyses were performed by pairwise comparisons of samples derived from the control strain grown in high-Pi or the Δ*mak-2* mutant grown in low-Pi conditions versus samples derived from the control strain grown in low-Pi conditions. Functional annotation of the differently expressed genes was performed according to Gene Ontology (Ashburner et al., 2000) and *N. crassa* Genome Database (Galagan et al., 2003). Gene Ontology term enrichment was performed using the BayGO algorithm (Vencio et al., 2006), and the significantly enriched terms (p-value < 0.05) were further analyzed.

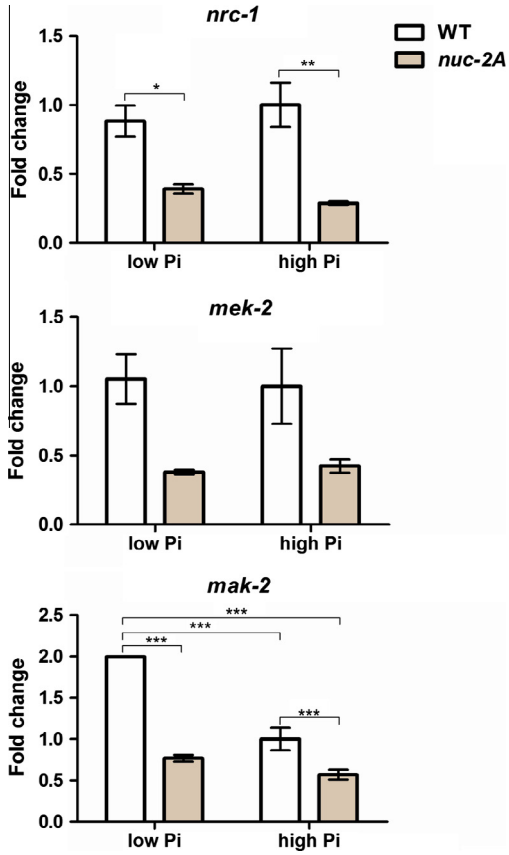

Fig. 1. Real-time PCR (qRT-PCR) analysis of *nrc-1* (NCU06182), *mek-2* (NCU04612), and *mak-2* (NCU02393) transcript levels in *nuc-2A* mutant strain of *N. crassa*. These genes were amplified from cDNA obtained from mycelia of control and *nuc-2A* mutant strains grown in low- and high-Pi liquid media. qRT-PCR data are representative of the average values ± standard deviation (SD) obtained from two independent experiments. Statistically significant values are indicated by asterisks: Tukey's *ad hoc* test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

2.4. Quantitative real-time PCR (qRT-PCR)

To validate microarray data, we performed qRT-PCR analysis of a subset of selected genes. Briefly, 1 μg of total RNA was used for cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (Invitrogen). qRT-PCR was performed using the StepOnePlus Real-time PCR System (Applied Biosystems). All experiments were carried out with two independent biological samples, and reactions were set in triplicate with the SYBR green PCR master mix (Applied Biosystems) by using 50 ng of cDNA, and 400 nM of each primer (Table S1), in a 12.5-μL final volume reaction. The PCR cycle was as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The $2^{-\Delta\Delta Ct}$ relative expression quantification method was used to calculate the fold change of each gene, using the actin gene as an endogenous control. Analysis was performed using the StepOne Software v2.2. As the reference sample, the control strain grown in high-Pi conditions was used to calculate the relative gene expression. Statistical analysis was performed using one-way ANOVA followed by the Tukey's *ad hoc* test. This analysis was performed using GraphPad Prism v 5.1 Software.

2.5. Microarray data

Raw whole genome microarray data were deposited in the Gene Expression Omnibus (GEO) database, http://www.ncbi.nlm.nih.gov/projects/geo/ (accession number GSE41806).

3. Results

A suppression subtractive hybridization library was previously constructed to identify transcripts up-regulated in the *nuc-2A* mutant strain of *N. crassa*, which was compared to the control strain, both strains grown under phosphate shortage. A number of genes were identified, including the *mak-2* gene, which codes for a MAPK

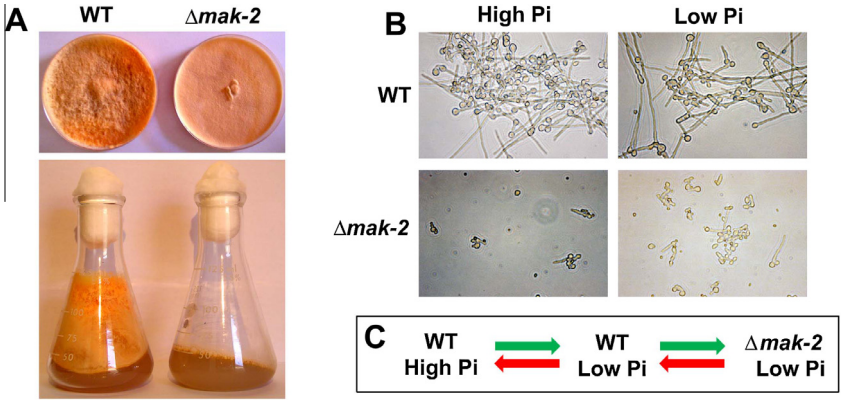

Fig. 2. Growth characteristics of the Δ*mak-2* mutant strain of *N. crassa*. (A) Δ*mak-2* mutant and control strains grown on Vogel's solid media; (B) Germination of the Δ*mak-2* mutant compared to the control strain in low- and high-Pi liquid media; (C) Schematic diagram showing the strategy used to compare the transcription profiles of the Δ*mak-2* mutant in low-Pi and control strains in both low- and high-Pi culture media.

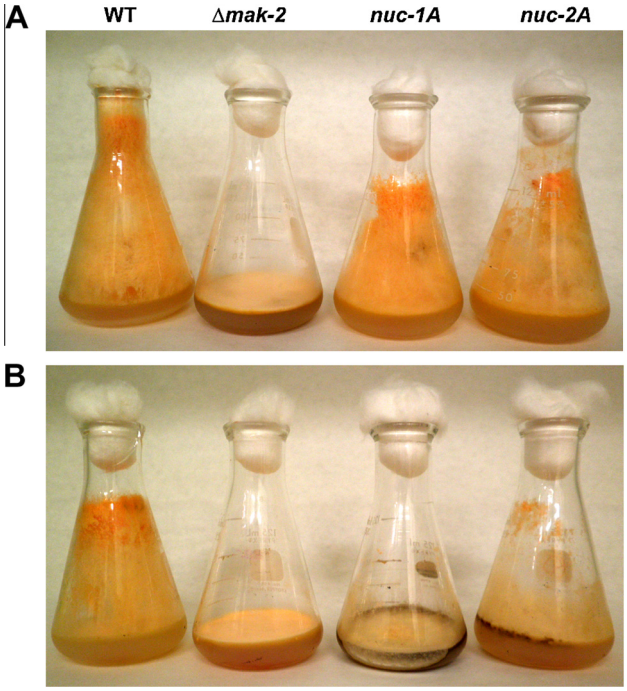

Fig. 3. Growth characteristics of Δ*mak-2*, *nuc-1A*, and *nuc-2A* mutant strains of *N. crassa* grown on high-Pi media (A) as compared to growth on 0.1% DNA as the sole Pi source (B).

(NCU02393) (Gras et al., 2007). This gene is involved in the sensing of a vast array of ambient stimuli in *N. crassa* (Li et al., 2005; Maerz et al., 2008; Pandey et al., 2004).

Here, similar transcription rates for *nrc-1*, and *mek-2* genes were observed in the control strain irrespective if growth has occurred in low- or high-Pi. However, when growth occurred in high-Pi the *mak-2* gene is down regulated. Similar transcription profiles for *nrc-1*, *mek-2*, and *mak-2* genes were observed in the *nuc-2A* mutant strain grown in either shortage or abundance of Pi (Fig. 1). However, the *nrc-1*, *mek-2*, and *mak-2* transcripts were down regulated in the *nuc-2A* mutant strain irrespective of growth has occurred under shortage or abundance of Pi as compared to the control strain (Fig. 1), suggesting that these three genes depend on the *nuc-2* gene activity to be properly expressed.

The Δ*mak-2* strain showed reduced growth on solid medium with abundant production of conidiophores close to the culture surface, which were morphologically similar to those produced by the control strain (Fig. 2A). Furthermore, reduced mycelial growth of the mutant strain was concomitant with reduced germination of conidia in liquid cultures (Fig. 2B). Growth characteristics of Δ*mak-2*, *nuc-1A*, and *nuc-2A* mutant strains of *N. crassa* in cultures supplied with DNA as the sole source of Pi as compared to the control strain grown in high-Pi cultures is shown in Fig. 3. The control and the Δ*mak-2* strains showed similar phenotypes when grown on DNA or on high-Pi cultures. Interestingly, the *nuc-2A* and *nuc-1A* mutant strains grown on DNA showed reduced aerial hyphal growth, a phenotype close to that shown by the Δ*mak-2* strain.

To further characterize the function of the *mak-2* gene in *N. crassa* regarding the phosphorus-sensing network, we compared the global gene expression profile of the *mak-2* knockout strain grown under Pi shortage with that of the control strain grown either in low- or high-Pi (Fig. 2C). Table S2 describes 912 unique genes with at least a 2.8-fold change in the expression levels, being 321 of them distributed into four clusters with overlapping genes

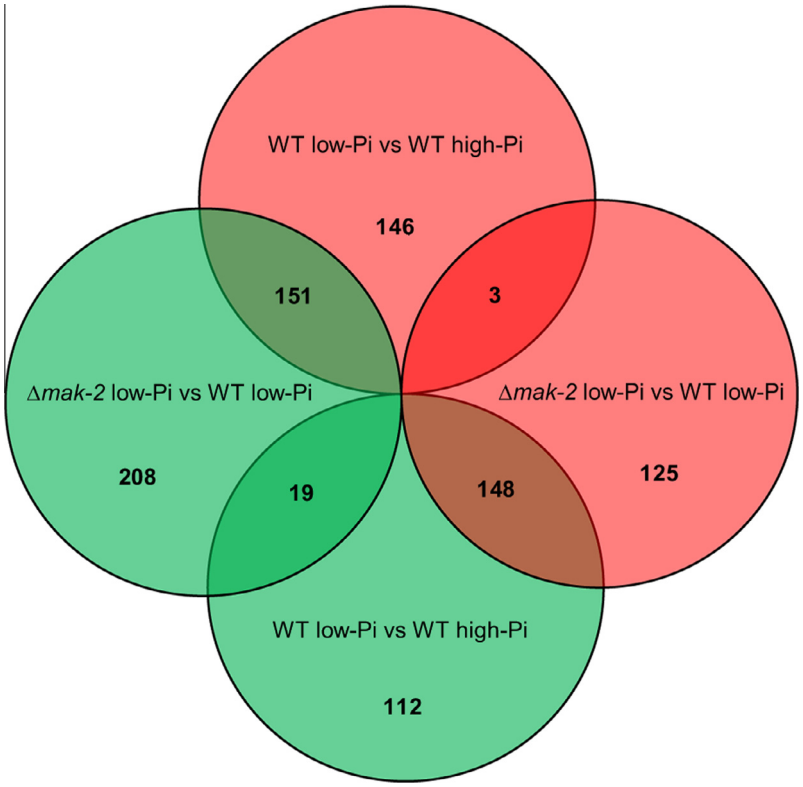

Fig. 4. Distribution of differentially expressed genes in *N. crassa*. The Venn diagram displays the extent of overlapping among differentially expressed genes in the Δ*mak-2* mutant and the control strains of *N. crassa*. Genes up-regulated and down-regulated are represented in red and green, respectively. The complete list of these genes is listed in Table S2.

Table 1
Genes possibly involved in phosphate acquisition pathway.

ID[a]	Gene	WT low-Pi versus WT high-Pi[b]	*Δmak-2* low-Pi versus WT low-Pi[b]	Gene product name	Functional annotation[c]
Phosphate transport, metabolism and regulation					
NCU09564	pho-4	24.93	−1.25	Phosphate-repressible phosphate permease	Signal transducer activity, phosphate metabolic process, phosphate transport
NCU08325	pho-5	46.85	−2.39	Inorganic phosphate transporter PHO84	Phosphate:hydrogen symporter activity, substrate-specific transmembrane transporter
NCU01376	pho-2	148.06	−4.89	Alkaline phosphatase	Phosphatase activity
NCU08643	pho-3	109.14	−13.64	Acid phosphatase	Phosphate acquisition
NCU09315	nuc-1	−1.35	1.21	Nuclease-1	Regulation of phosphorus utilization, phosphorus acquisition-controlling protein
NCU11426	nuc-2	3.97	−1.56	Nuclease-2, ankyrin repeat protein NUC-2	Phosphate signal transduction pathway, response to light stimulus
NCU01738	preg	1.18	2.22	Negative regulatory factor	Phosphatase regulation, regulatory protein
NCU07580	pgov	−1.08	1.28	Cyclin-dependent protein kinase	Phosphate metabolic process, protein phosphorylation
NCU07027		−1.48	−3.92	Glycogen phosphorylase	Glycogen degradation
NCU01632		−6.15	3.84	Pentafunctional AROM polypeptide	Aromatic amino acid family biosynthetic process, shikimate kinase activity
NCU03061	rli-1	1.89	2.99	Translation initiation factor RLI1	Translational initiation, iron ion binding
NCU02075	hsp70	−6.15	3.84	Heat shock protein 70	Unfolded protein binding, regulation of translational fidelity
Post-translational modification					
NCU00034	ste-14	−1.64	12.21	Prenyl cysteine carboxyl methyltransferase Ste14	Response to organic substance
NCU05999	ram-1	−1.13	4.66	Caax farnesyltransferase beta subunit Ram1	Response to stress, response to inorganic substance
NCU01388	mnt-1	−1.37	2.55	Alpha-1,2-mannosyltransferase	Hyphal growth, conidium formation, sporocarp development involved in sexual reproduction
NCU03632	ram-2	−1.24	2.43	Farnesyltransferase/ geranylgeranyltransferase type I alpha subunit	Signaling
NCU03191	rab-1	−2.14	−1.21	Related to rab geranylgeranyl transferase component A	Signaling
Mapk signaling					
NCU06182	nrc-1	1.05	−1.32	MAPKK kinase nonrepressor of conidiation-1	Spore germination, conidium formation, hyphal growth, sporocarp development involved in sexual reproduction
NCU04612	mek-2	1.39	1.56	Mitogen activated protein kinase kinase 2	Hyphal growth, sporocarp development involved in sexual reproduction, ascospore formation, conidiophore development
NCU02393	mak-2	1.13	−19.70	Mitogen-activated protein kinase 2	Hyphal growth, chemotropism, protein phosphorylation, conidium formation, spore germination, regulation of growth rate
NCU03071	os-4	1.37	1.16	MAP kinase kinase kinase sskb osmotic sensitive-4	Response to light stimulus, sporocarp development involved in sexual reproduction, response to salt stress, glycerol biosynthetic process
NCU00587	os-5	−1.39	−1.13	Mitogen activated protein kinase kinase, osmotic sensitive-5	Sporocarp development involved in sexual reproduction, response to osmotic stress, ascospore formation, conidium formation
NCU07024	os-2	−2.22	−1.04	Mitogen-activated protein kinase osmotic sensitive-2	Response to osmotic stress, cellular response to glucose starvation
NCU02234	mik-1	−1.01	−1.10	MAP kinase kinase kinase	Conidium formation, hypal growth, ascospore formation, conidiophore development
NCU06419	mek-1	−1.14	−2.17	MAP kinase kinase	Hyphal growth, regulation of melanin biosynthetic process, conidium formation, sporocarp development involved in sexual reproduction
NCU09842	mak-1	−1.32	1.11	Mitogen-activated protein kinase MKC1	Ascospore formation, hyphal growth, sporocarp development involved in sexual reproduction, regulation of growth rate

[a] Genes in bold were analyzed by quantitative Real-time PCR.
[b] Gene expression values are indicated as fold change between samples. Not significantly differentially expressed genes are indicated by gray color.
[c] Functional annotation according to Gene Ontology (Ashburner et al., 2000) and *Neurospora crassa* Genome Database (Galagan et al., 2003).

(Fig. 4). These genes were functionally annotated according to Gene Ontology and *N. crassa* Genome Project, and an enrichment Bayesian analysis identified over-represented modulated categories (Table S3). Some of these gene categories, such as those representing structural constituents of ribosome, translational elongation, RNA binding, reproduction, positive regulation of growth rate, and protein binding were down regulated in the control strain grown in low-Pi versus high-Pi, a physiological condition in which *nuc-1* gene is functional (Fig. S1). Thus, down-regulation of genes in the control strain grown in low-Pi is presumed to be dependent on NUC-1. These functional gene categories were up-regulated in the mutant Δ*mak-2* versus control, both strains grown in low-Pi. On the other hand, those gene categories representing for example responsiveness to light stimulus and cell stress revealed an opposite expression profile i.e., these genes were down-regulated in Δ*mak-2* versus control, both strains grown in low-Pi, and up-regulated in the control grown in low-Pi versus high-Pi. (Fig. S1). Thus, transcription profiling of these gene categories in the Δ*mak-2* strain grown under Pi shortage was similar to that of the control strain grown in high-Pi cultures suggesting that *nuc-1* gene is non-functional in the Δ*mak-2* strain.

A list of differentially expressed genes related to phosphate metabolism, and regulation, MAPK signaling, protein prenylations and biosynthesis of metabolites such as sterols, dolichols, and ubiquinones are shown in Table 1. Genes potentially modulated by NUC-1, showing the NUC-1 DNA binding domain 5′-CACGTG-3′ in

Table 2
Genes potentially modulated by NUC-1 protein.

ID[a]	Gene	WT low-Pi versus WT high-Pi[b]	*Δmak-2* low-Pi versus WT low-Pi[b]	Gene product name	Functional annotation[c]
NCU01376	*pho-2*	147.79	−4.88	Alkaline phosphatase	Phosphatase activity
NCU08643	*pho-3*	108.99	−13.63	Acid phosphatase	Phosphate acquisition
NCU07484	*gh18-8*	42.03	−48.44	Chitinase	Response to nutrient levels
NCU09904		22.50	−40.13	Glucan 1,3-beta-glucosidase	Hydrolase activity
NCU05980		16.73	−49.28	Carboxypeptidase S1	Hydrolase activity
NCU00790	*hak-1*	13.56	−28.49	High affinity potassium transporter-1	Response to light stimulus, potassium ion transmembrane transporter activity
NCU09788		11.80	−6.41	Endonuclease/ exonuclease/ phosphatase	
NCU04533		4.77	−4.03	Abundant perithecial protein	
NCU08356		4.34	−3.37	Acetamidase	Hydrolase activity, acting on carbon–nitrogen (but not peptide) bonds, in linear amides, mitochondrial outer membrane
NCU09791		3.01	−5.27	Beta-1,3-exoglucanase	Hydrolase activity
NCU07737		2.92	−21.61	Salicylate hydroxylase	Salicylate degradation
NCU11426	*nuc-2*	3.97	−1.56	Nuclease-2, ankyrin repeat protein NUC-2	Phosphate signal transduction pathway, response to light stimulus

[a] These genes present the NUC-1 DNA binding consensus 5′-CACGTG-3′ (Peleg and Metzenberg, 1994) in their promoter region, except *nuc-2* that shows the presumed consensus 5′-CACG**A**G-3′ for NUC-1 binding. Genes in bold were analyzed by quantitative Real-time PCR.
[b] Gene expression values are indicated as fold change between samples.
[c] Functional annotation according to Gene Ontology (Ashburner et al., 2000) and *Neurospora crassa* Genome Database (Galagan et al., 2003).

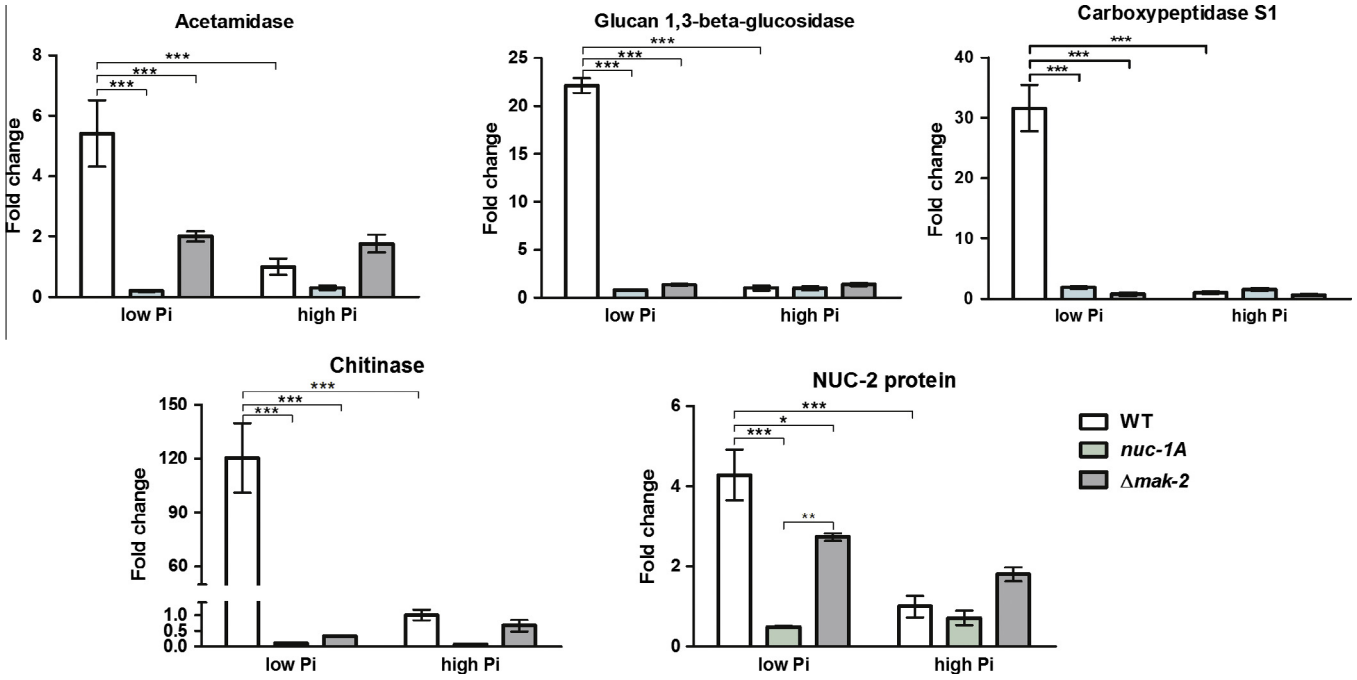

Fig. 5. Validation of differentially expressed genes using quantitative Real-time PCR (qRT–PCR). Five genes potentially modulated by the *nuc-1* gene were amplified from cDNA obtained from mycelia of the control, Δ*mak-2*, and *nuc-1A* mutant strains of *N. crassa* grown in high- and low-Pi media. These genes code for acetamidase (NCU08356); glucan 1,3-beta glucosidase (NCU09904); carboxypeptidase S1 (NCU05980); chitinase (NCU07484), and NUC-2 protein (NCU11426, *nuc-2*). These genes show the DNA binding consensus 5′-CACGTG-3′ for NUC-1, except *nuc-2* that shows the presumed consensus 5′-CACG**A**G-3′ for NUC-1. qRT-PCR data are representative of the average values ± standard deviation (SD) obtained from two independent experiments. Statistically significant values are indicated by asterisks: Tukey's *ad hoc* test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

their promoter region (Peleg and Metzenberg, 1994), except *nuc-2* gene that showed the presumed binding consensus 5′-CACG**A**G-3′ for NUC-1, are listed in Table 2. Some of these genes, shown in Fig. 5, were up-regulated in the control strain grown in low Pi, and down-regulated in the *nuc-1A* and *mak-2* strains, suggesting the involvement of MAK-2 in the functioning of NUC-1.

Expression analysis by qRT-PCR of 18 selected genes, including the *nuc-2* gene, using independent RNA samples and described in Tables 1 and 2 in bold, validated the microarray data (Figs. 1, 5 and 6). Statistical significance was achieved for all genes, except for the *preg* gene, according to variance analysis (ANOVA) ($P < 0.05$). Furthermore, the expression levels obtained in the microarray experiments and in qRT-PCR assays were compared regarding to the $\log_2$ fold change in the expression levels in the control strain grown in low-Pi versus its growth in high-Pi conditions. The same comparison was performed for the expression

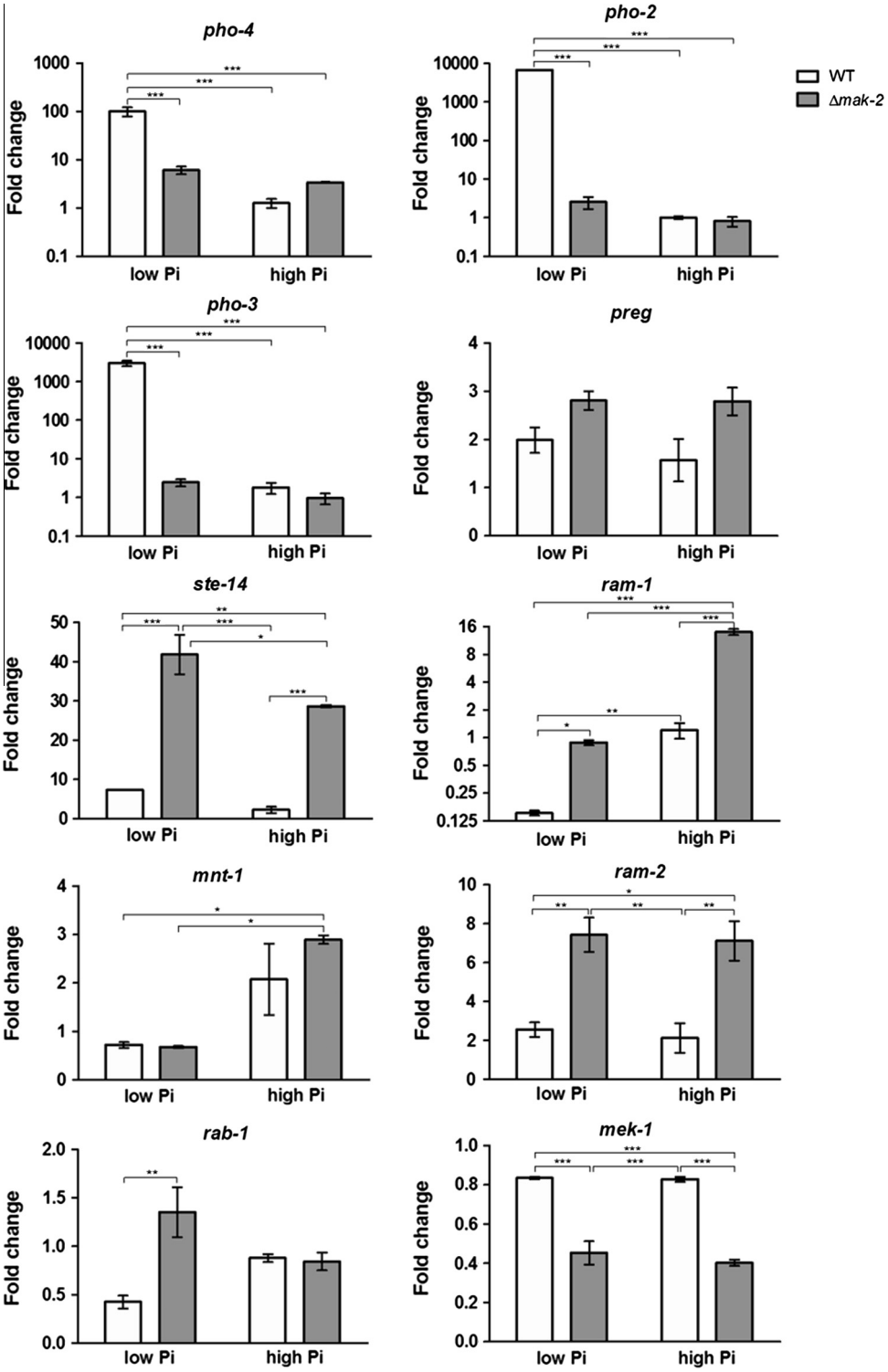

Fig. 6. Validation of differentially expressed genes using quantitative Real-time PCR (qRT–PCR). Ten genes potentially modulated in response to Pi and/or *mak-2* mutation were amplified from cDNA obtained from mycelia of the control and Δ*mak-2* mutant strains of *N. crassa* grown in high- and low-Pi liquid media [*pho-4* (NUC09564); *pho-2* (NCU01376); *pho-3* (NCU08643); *preg* (NCU01738); *ste-14* (NCU00034); *ram-1* (NCU05999); *mnt-1* (NCU01388); *ram-2* (NCU03632); *rab-1* (NCU03191) and *mek-1* (NCU06419)]. qRT-PCR data are representative of the average values ± standard deviation (SD) obtained from two independent experiments. Statistically significant values are indicated by asterisks: Tukey's *ad hoc* test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

levels in the Δ*mak-2* mutant strain versus the control strain, with both strains grown in low-Pi conditions (Table 3). Pearson correlation obtained between microarray and qRT-PCR data was statistically significant, $r = 0.87$, $P > 0.001$, reinforcing the reliability of large-scale microarray transcriptional profiling.

4. Discussion

The availability of Pi is sensed in *N. crassa* by the *nuc-2* gene, whose transcription is de-repressed in the mycelium grown under Pi shortage and is repressed in the presence of excess Pi (Fig. 5). It

Table 3
Comparison between gene expression values obtained by microarray and Real-time PCR analysis.

ID	Gene product name	Microarray		qRT-PCR	
		WT low-Pi versus WT high-Pi	*Δmak-2* low-Pi versus WT low-Pi	WT low-Pi versus WT high-Pi	*Δmak-2* low-Pi versus WT low-Pi
NCU06182	MAPKK kinase nonrepressor of conidiation-1 NRC-1	1.05	−1.32	−1.13	ND
NUC04612	Mitogen activated protein kinase kinase 2 MEK-2	1.39	1.56	1.05	ND
NCU02393	Mitogen-activated protein kinase-2 MAK-2	1.13	−19.70	2.21	ND
NCU09564	Phosphate-repressible phosphate permease PHO-4	24.93	−1.25	70.52	−14.62
NCU01376	Alkaline phosphatase PHO-2	148.06	−4.89	6841.04	−2646.74
NCU08643	Acid phosphatase PHO-3	109.14	−13.64	1640.59	−1217.75
NCU01738	Negative regulatory factor PREG	1.18	2.22	1.27	1.41
NCU00034	Prenyl cysteine carboxyl methyltransferase STE-14	−1.64	12.21	3.29	6.15
NCU05999	CaaX farnesyltransferase beta subunit RAM-1	−1.13	4.66	−8.17	6.06
NCU01388	Alpha-1,2-mannosyltransferase MNT-1	−1.37	2.55	−3.20	−1.03
NCU03632	Farnesyltransferase/geranylgeranyltransferase alpha subunit RAM-2	−1.24	2.43	1.21	2.91
NCU03191	Rab geranylgeranyl transferase component A RAB-1	−2.14	−1.21	−2.25	3.51
NCU06419	MAP kinase kinase MEK-1	−1.14	−2.17	3.34	−3.36
NCU08356	Acetamidase	4.32	−3.36	5.41	−2.69
NCU09904	Glucan 1,3-beta-glucosidase	41.93	−39.95	22.12	−16.49
NCU05980	Carboxypeptidase S1	22.47	−49.18	31.59	−42.18
NCU07484	Chitinase	41.93	−48.50	120.40	−355.40
NCU11426	Nuclease-2 ankyrin repeat protein NUC-2	3.97	−1.56	4.28	−1.56

Gene expression values are indicated as fold change between samples. Not significantly differentially expressed genes are indicated by gray color. ND: Not determined.

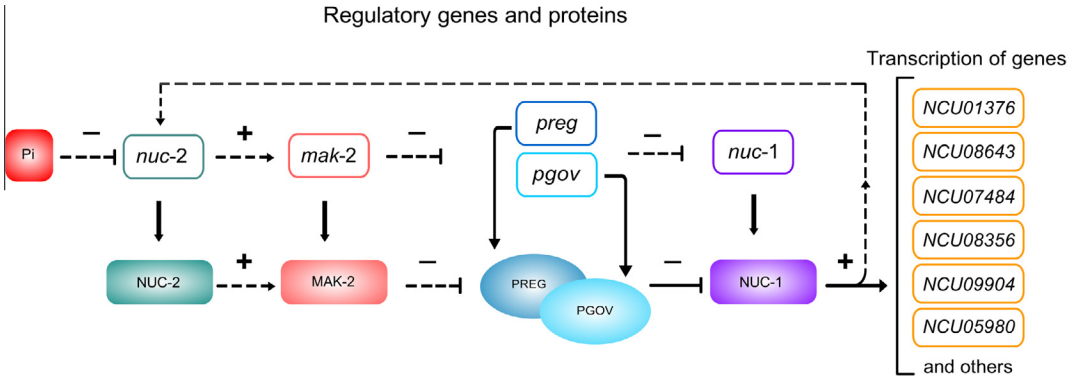

Fig. 7. Extended model for the hierarchical control of phosphorus acquisition enzyme synthesis in *N. crassa* (Leal et al., 2007; Metzenberg, 1979; Peleg et al., 1996a). Activation and inhibition are marked by positive and negative signs, respectively. Broken lines represent the action of phosphate or genes presumably through metabolic pathways that have not yet been fully elucidated. The *loci* ID code for alkaline phosphatase (NCU01376); acid phosphatase (NCU08643); chitinase (NCU07484); acetamidase (NCU08356); glucan 1,3-beta-glucosidase (NCU09904) and carboxypeptidase S1 (NCU05980). Other signaling pathways in which the MAK-2 protein is involved are not represented in the proposed model.

has been extensively demonstrated that NUC-2 protein transmits a metabolic signal downstream the hierarchical regulatory pathway that, under Pi shortage, interacts with the PREG-PGOV complex, allowing the translocation of the transcription factor NUC-1 into the nucleus thus activating the synthesis of Pi-repressible enzymes (Table 1). On the other hand, in the presence of excess Pi, transcription of the *nuc-2* gene is down-regulated; the amount of NUC-2 protein is lowered in the cell, and the interaction of NUC-1 with the PREG-PGOV complex is predominant, inhibiting the translocation of the NUC-1 protein into the nucleus. Under these conditions, it has been shown that the Pi-repressible phosphatase genes are not transcribed (Leal et al., 2009; Metzenberg, 1979; Peleg et al., 1996a). Transcription of the *preg*, *pgov*, and *nuc-1* genes are probably not responsive to changes in the extracellular Pi levels (Table 1), reinforcing that Pi availability is primarily sensed by the *nuc-2* gene, whose transcription is regulated in response to ambient Pi changes (Peleg et al., 1996b). Thus, a loss-of-function mutation in the *nuc-2* gene, as that in the *nuc-2A* mutant, makes the mutant strain unable to sense Pi levels i.e., irrespective of the extracellular Pi concentration this sort of mutant does not transcribe the Pi-repressible phosphatases. Moreover, similar transcription rates for *nrc-1*, *mek-2*, and *mak-2* were observed in the *nuc-2A* mutant strain in which, irrespective of the ambient Pi levels these genes were apparently down-regulated as compared to the control, suggesting that transcription rate of these MAPKs is dependent on NUC-2 activity under these experimental conditions (Fig. 1).

The transcriptional profiling of the *mak-2* knockout revealed several genes that are repressed in the Δ*mak-2* strain grown either in low- or high-Pi media; thus behaving as grown in high-Pi cultures regarding the Pi-repressible genes. Some of them are those genes coding for acetamidase, chitinase, acid and alkaline phosphatases, carboxipeptidase S1, glucan 1,3-beta-glucosidase, and NUC-2 and PHO-4 proteins (Figs. 5 and 6). A similar phenotype was previously observed in both the *nuc-2A* and *nuc-1A* mutant strains for the transcription of the genes coding for the Pi-repressible phosphatases (Ishikawa et al., 1969; Metzenberg, 1979). Indeed, the *mak-2* knockout revealed a large number of gene categories that are up- or down-regulated in low-Pi, whose transcription profiling is similar to that of the wild type grown in high-Pi (Fig. S1). These genes represent a vast array of metabolic reactions involved in a number physiological processes, which are not surprising since during growth nutrient signaling and starvation, conidial germination, and hyphal development, among others, are in progress. Particularly interesting is the

earlier transcription activation of gene categories responsive to light stimulus since conidia were germinated for 5 h in an orbital shaker (Fig. 2). It is worth noting that only few genes among those previously identified in the *N. crassa* Δ*mak*-2 strain (Li et al., 2005) and in the *Podospora anserina* Δ*PaMpk2* strain (Bidard et al., 2012), a gene orthologous to *mak-2*, were revealed in our microarray experiments (Table S4). These results are not surprising because in addition to different organisms, the design of our experiments is also different from these previous ones, making difficult a straightforward comparative analysis. However, down-regulation of the gene encoding for the hydrophobin precursor (NCU08457) may, for example, explain the lack of aerial hyphae in the Δ*mak-2* mutant as previously proposed for *Podospora* (Bidard et al., 2012).

In an extended version for the Pi-signaling network we are proposing that MAK-2 and NUC-2 proteins are functional in *N. crassa* cultured under Pi shortage and are non-functional under abundant Pi conditions regarding transcription of the Pi-repressible genes. *In silico* prediction of protein–protein interactions available at the "*Neurospora crassa* Protein Interactome Database" (Wang et al., 2011) revealed a putative interaction of MAK-2 with PREG and PGOV, but not with NUC-2 and NUC-1. MAK-2 possibly inhibits the functioning of the PREG-PGOV complex allowing the translocation of the transcription factor NUC-1 into the nucleus thus activating the synthesis of the Pi-repressible enzymes (Fig. 7). Also, when MAK-2 is non-functional, abundant Pi for instance, the PREG-PGOV complex interacts with NUC-1 inhibiting its translocation into the nucleus. Moreover, transcription of the *nuc-2* gene is down-regulated (at least 8-fold changes) in the *nuc-1A* mutant strain grown under Pi shortage (Fig. 5). Thus, transcription of the *nuc-2* gene is modulated by the transcriptional regulator NUC-1, and *nuc-2* feedback transcription probably amplifies the metabolic signal that NUC-2 protein transmits downstream of the hierarchical regulatory pathway.

A set of other genes responds differently when the *mak-2* gene is functional in both low- and high-Pi cultures. Similar profiles of *ste-14* (Anderson et al., 2005; Griggs et al., 2010), *ram-2* (Kuranda et al., 2010; Nakayama et al., 2011; Song and White, 2003), and *mek-1* (Maerz et al., 2008) transcripts are observed in the control strain in the presence of low- or high-Pi concentrations; thus, transcription of these genes is not responsive to extracellular Pi changes (Fig. 6). However, the *ste-14* and the *ram-2* genes are down-regulated, whereas the *mek-1* gene is up-regulated when the *mak-2* gene is functional. Thus, transcription of these three genes occurs independently of *nuc-2* and *nuc-1* functioning, suggesting an alternative metabolic route for MAK-2 function. Transcription of the *mnt-1* (Bowman et al., 2005), *rab-1* (Liu and Storrie, 2012; Zerial and McBride, 2001), and *ram-1* (He et al., 1991) genes is apparently responsive to extracellular Pi changes, being down regulated when the *nuc-2* gene is functional. Transcription of the *mnt-1* gene is not responsive to the *mak*-2 gene, whereas transcription of *rab-1* is responsive to *mak-2* gene. Other genes revealed in our microarray experiments were negatively modulated by the product of the *mak*-2 gene (Fig. 6). The *ste-14*, *ram-1*, and *ram-2* genes are components of the isoprenoid pathway, which is essential for the survival of fungi and plays a role in protein prenylations and the biosynthesis of metabolites such as the sterols, dolichols, and ubiquinones (Kuranda et al., 2010; Kuzuyama and Seto, 2012; Nakayama et al., 2011). Rab proteins, a class of abundant proteins that includes the Ras superfamily of small GTPases, are prenylated. This posttranslational modification is also regarded as important in protein–protein interactions, such as those that facilitate protein trafficking and sub cellular localization (Liu and Storrie, 2012; Zerial and McBride, 2001).

5. Conclusions

The genome wide microarray transcription profiling was successfully used to identify down- and up-regulated genes in the *N. crassa* Δ*mak-2* strain grown under limited Pi as compared to the control strain grown either in low- or high-Pi media. Our results provide conclusive evidence that the *mak-2* gene participates in the Pi-signaling pathway in *N. crassa* in addition to its involvement in other metabolic routes such as the isoprenylation pathway. In an extended version for the Pi-signaling network we are proposing that MAK-2 and NUC-2 proteins are functional when *N. crassa* is cultured under Pi shortage and are non-functional under abundant Pi conditions regarding transcription of the Pi-repressible genes. Thus, MAK-2 possibly inhibits the functioning of the PREG-PGOV complex allowing the translocation of the transcription factor NUC-1 into the nucleus; this activating the synthesis of the Pi-repressible enzymes. We identified presumed interaction of MAK-2 with PREG and PGOV, but not with NUC-2 and NUC-1. Furthermore, when MAK-2 is non-functional, abundant Pi for instance, the PREG-PGOV complex interacts with NUC-1 inhibiting its translocation into the nucleus. Remarkably, transcription of the *nuc-2* gene is modulated by the transcriptional regulator NUC-1, and *nuc-2* feedback transcription probably amplifies the metabolic signal that NUC-2 protein transmits downstream of the hierarchical regulatory pathway. We also suggest that the *mak-2* gene facilitates protein trafficking by negatively modulating some genes such as *rab-1* under Pi-shortage. The results presented here reveal novel aspects of the phosphorus-sensing network, emphasizing the complexity of the metabolic modulation exerted by the MAPK MAK-2 in *N. crassa*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2013.05.007.

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