Chapter 15
Assimilatory Nitrate Reduction in *Hansenula polymorpha*

Beatrice Rossi and Enrico Berardi

Contents

Abstract In the last decade, the yeast *Hansenula polymorpha* (syn.: *Pichia angusta)* has become an excellent experimental model for genetic and molecular investigations of nitrate assimilation, a subject traditionally investigated in plants, filamentous fungi and bacteria. Among other advantages, *H. polymorpha* offers classical and molecular genetic tools, as well as the availability of genomic sequence data.

Assimilative nitrate metabolism in *H. polymorpha* has an enzymological layout that is similar to other fungal species, and undergoes nitrogen metabolite repression elicited by preferred nitrogen sources such as glutamine. Genes involved in nitrate assimilation are clustered and independently transcribed. The information that puzzles is the presence of two homologous, albeit different, transcriptional activators acting upon the nitrate cluster genes, as all other known fungal nitrate assimilatory pathways have only one activator of this family. Recent work enables a first outline of the interplay between these two activators to be depicted, and suggests that one of them plays a central role in chromatin remodelling within the cluster.

The information, which has recently emerged regarding complex post-translational down-regulatory mechanism acting upon the major nitrate transporter suggests that this protein plays a central role in the regulation of nitrate assimilation.

Nitrogen metabolite repression acting upon nitrate assimilative genes is also being investigated through the isolation and characterisation of *H. polymorpha Nmr* mutants. These studies have suggested that the repression mechanisms are mediated by several interacting factors in this organism, which are also believed to

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participate in nitrogen metabolite repression of other metabolic pathways. All these are involved in the utilisation of secondary nitrogen sources such as arginine, methylamine, urea and asparagine.

Keywords *Pichia angusta*, nitrate assimilation, gene cluster, transcriptional regulation, *trans*-activator, post-translational regulation, chromatin remodelling, nitrogen metabolite repression

15.1 Introduction

Assimilative nitrate reduction is the major pathway converting inorganic nitrogen to organic forms. It has been estimated that more than 2–10% megatons of organic nitrogen per year are produced by nitrate assimilating organisms, a variety of species including bacteria, fungi, algae and plants (Guerrero et al., 1981). According to Kay et al. (1990), as much as 25% of the energy of photosynthesis is consumed in driving nitrate assimilation.

For this reason, nitrate assimilation is crucially related to aspects of great relevance and impact for human life and biosphere equilibrium (e.g. crop yield improvement, concern over the use of nitrate fertilizers, costly in terms of production and in terms of ecological and toxicological impact).

The enzymological layout of assimilative nitrate metabolism (Fig. 15.1) is similar in all fungal species studied so far. It consists of a two-step reduction of nitrate to ammonia, catalysed by the sequential action of nitrate reductase (NR) and nitrite reductase (NiR). NR catalyses the two-electron reduction of nitrate to nitrite; it is a complex multicentre enzyme that in eukaryotes uses NAD(P)H as electron donor

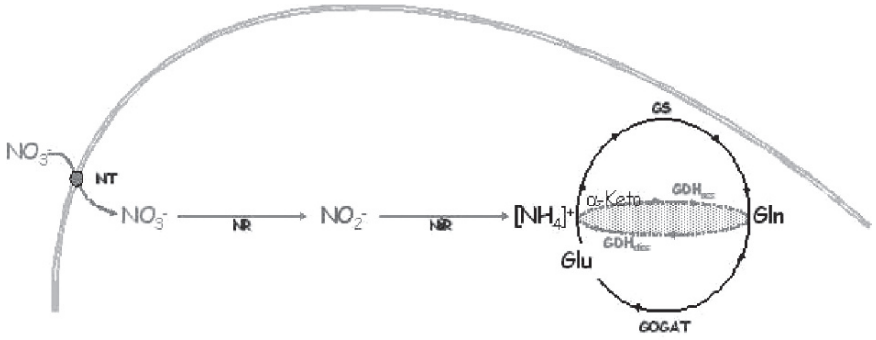

Fig. 15.1 Enzymological layout of assimilative nitrate metabolism in fungi. The two-step reduction of nitrate to ammonia is catalysed by the sequential action of nitrate reductase (NR) and nitrite reductase (NiR). Glutamine Syntetase (GS) fixes ammonia to an α-ketoglutarate skeleton to form glutamine, which can undergo a second ammonia fixation (catalysed by GOGAT) to form Glutamate (Glu)

and contains FAD, ferrohaem and molybdopterin prosthetic groups. It is considered to be a limiting factor for growth, development and protein production in all nitrate-assimilating organisms. NiR catalyses the six-electron reduction of nitrite to ammonia; it is also a multicentre enzyme that in plants uses reduced ferredoxin as electron donor, whilst in bacteria and fungi uses NAD(P)H (Campbell and Kinghorn, 1990; and references therein).

Traditionally, genetic and molecular studies of nitrate assimilation have been done mostly in plants, filamentous fungi and bacteria (Wray and Kinghorn, 1989, and reviews therein). Although a large number of nitrate-assimilating yeasts exist (Barnett et al., 1984), only in the last fifteen years has a nitrate assimilating yeast become the object of molecular investigations, namely *Hansenula polymorpha* (syn.: *Pichia angusta)*. This ascomycete is an excellent experimental model, both for transport studies and for understanding regulatory mechanisms.

In most nitrate assimilating microbial species, nitrate is usually one of many possible nitrogenous compounds that can be utilised, and *H. polymorpha* is no exception. It is believed that this metabolic versatility is an adaptive strategy possibly evolved to confer species better adaptability in environments with variable substrate composition. As a consequence, complex regulatory circuits exist governing the assimilation of nitrogen compounds and the priority a given cell confers to each different nitrogen compound, in case two or more of these compounds occur simultaneously.

All biochemical and genetic studies related to nitrate in *H. polymorpha* descend from our laboratory in Ancona and from J. Siverio's group at La Laguna (Machin et al., 2004; Navarro et al., 2003, 2006; Pignocchi et al., 1998; Rossi et al., 2005; Serrani et al., 2001; Serrani and Berardi, 2005; Siverio, 2002 and references therein).

As for other microbial heterotrophs, also for *H. polymorpha,* nitrogen sources are conventionally classified as *primary* or *secondary* sources. The former are preferentially utilised and capable of sustaining high growth rates, as their incorporation in the living matter is energetically cheap (glutamine, glutamate, ammonia). The latter, which include nitrates and nitrites are only utilised when primary sources are absent (see Pignocchi et al., 1998). In the cell, the ability to switch on the metabolisms necessary for the utilisation of secondary nitrogen sources is rapidly developed, in response to the absence of *primary* sources and in the presence of a specific secondary source e.g. nitrate, that is regarded as *inducer* (Avila et al., 1995; Avila et al., 2002; Brito et al., 1996; Pignocchi et al., 1998; Serrani et al., 2001; Rossi et al., 2005).

A general picture of regulation of nitrogen source utilisation in fungi was sketched out as early as 1973 by Arst and Cove (1973) through imaginative studies on *Aspergillus* mutants (Fig. 15.2).

Any structural gene involved in the utilisation of a secondary nitrogen source was proposed to undergo dual control: (i) nitrogen metabolite repression, acting upon a trans-activating factor with general action (i.e. over all *Nmr*-sensitive genes), in a way that nitrogen depletion would release repression; (ii) induction, acting via factors that are specific for each secondary source utilisation (e.g. nitrate).

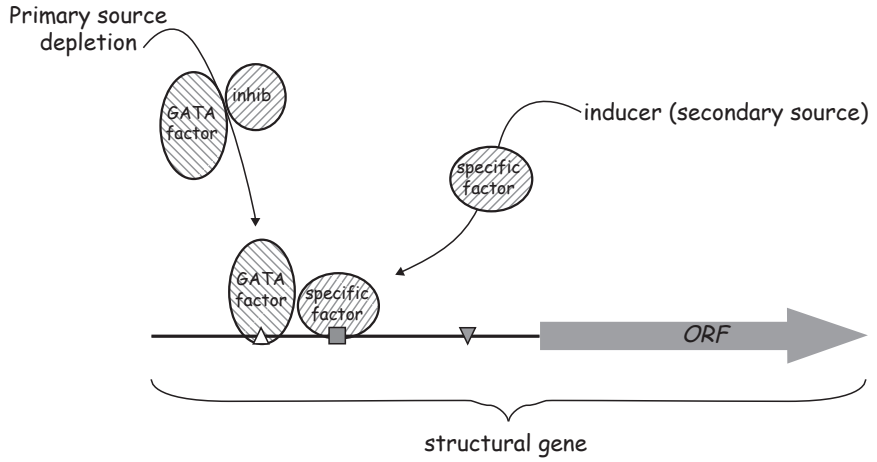

Fig. 15.2 General picture of regulation of nitrogen source utilisation in fungi

During the years further work (both with *Aspergillus* and *Neurospora)* confirmed and enriched this general picture, and new molecular players appeared, such as the specific inducer NirA, acting at different levels and enabling a much more complex picture to be sketched out (Bernreiter et al., 2007; Berger et al., 2006; Burger et al., 1991a, b; Caddick et al., 2006; Feng et al., 1996, 1998; Fu et al., 1995; Marzluf, 1997; Narendja et al., 2002; Strauss et al., 1998; Yuan et al., 1991).

15.2 Nitrate Assimilation in *H. polymorpha*

As said, nitrate assimilation in *H. polymorpha* is similar to that of other fungi, such as *Aspergillus nidulans* and *Neurospora crassa*. It requires *de novo* synthesis of one or more transporters (e.g. Ynt1p; Pérez et al., 1997), of NR (nitrate reductase i.e. NADPH: nitrate oxidoreductase, EC 1.6.6.3; Avila et al., 1995) and of NiR (nitrite reductase i.e. NADPH: nitrite oxidoreductase, EC 1.6.6.4; Brito et al., 1996). Once taken up, nitrate is firstly reduced to nitrite, then to ammonia, so as to enter cellular metabolic circuits (Fig. 15.3).

15.2.1 Nitrate Transport

As in other organisms (e.g. *Aspergillus nidulans; Chlamydomonas reinhardtii*; Unkles et al., 1991; Navarro et al., 2000; Galván et al., 1996), it has been suggested that *H. polymorpha* has more than one nitrate transporters (Machín et al., 2000; Machín et al., 2004), although only one transporter gene has been isolated so far

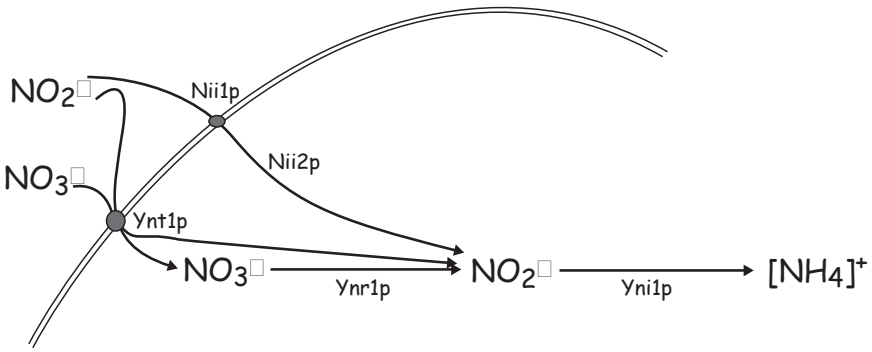

Fig. 15.3 Assimilative nitrate and nitrite reduction in *H. polymorpha*. Nitrate enters the cell *via* transporter Ynt1p, which is also involved in nitrite uptake. Nitrite is also taken up by specific transporter Nii1p. Nitrate reduction is catalysed by nitrate reductase Ynr1p. Nitrite reduction is catalysed by nitrite reductase Yni1p.)

*(YNT1,*Pérez et al., 1997). *YNT1* encodes a high affinity nitrate transporter (K_m 2–3 μM; V_{max} 0.5 nmol/NO_3/min/mg cell), believed to represent the most relevant one in quantitative terms (Machín et al., 2000). Ynt1p appears to be also a nitrite transporter (Machín et al., 2004) and belongs to group II of NNP family of nitrate transporters (nitrate nitrite porter; Forde, 2000), the same as *Asp. nidulans crnA* (*nrtA)* e *nrtB* (Unkles et al., 1991, 2001).

All group II members exhibits high sequence similarities and share the same membrane topology. In particular, a distinctive feature is a long hydrophilic sequence (90 aa) spanning between the sixth and seventh *trans*-membrane domain, and a C-terminal end shorter than that of other groups (*vide* Forde, 2000). Ynt1p plays a central role in the regulation of nitrate assimilation through a complex post-translational down-regulatory mechanism (Navarro et al., 2003, 2006). In the presence of primary nitrogen sources *Ynt1p-ubiquitin conjugates* are formed, associated to rapid vacuolar Ynt1p degradation. This fact brings about a quick decrease of nitrates transported into the cytoplasm, a reduction in the induction level of other genes involved in nitrate assimilation and, ultimately, a rapid modulation of nitrate uptake in response to nitrogen sources present in the medium. Navarro et al. have recently been shown that the central hydrophilic domain of Ynt1p harbours *PEST-like sequences,* deletion of which impedes the down-regulatory process of Ynt1p. In particular, site-directed mutagenesis showed that Lys-253 and Lys-270 within these domains are specifically involved in Ynt1p vacuole intake and degradation (Navarro et al., 2006). Interestingly, Ynt1p is degraded in response to glutamine, independently of glutamine repression, as shown by repression defective mutants (Navarro et al., 2006). Therefore, response to primary nitrogen sources seems to occur in two steps. A short term one, whereby Ynt1p down-regulation lowers nitrate intake by decreasing the number of transporter molecules; a long term one, whereby low intracellular nitrate levels would reduce the induction of nitrate assimilating gene expression (Navarro et al., 2006).

H. polymorpha is capable of utilising nitrite. This ion can enter the cell either via Ynt1p or via a different transporter. In our laboratory two complementation groups of mutants specifically impaired in nitrite assimilation *(nitrite$^-$ nitrate$^+$)* have been isolated, namely *Nii1* and *Nii2*. Experimental data suggests that *NII1* may represent a specific nitrite transporter, whereas complementation work led to the isolation of *NII2*. This gene is predicted to encode a membrane protein, homologue to Pho (Serrani and Berardi, 2005). Although Nii2p role remains unknown, by analogy to Pho, it can be speculated that it plays an accessory role in the maturation of the true transporter. It has been shown that nitrite specific system prevails at pH 4, whereas Ynt1p overcomes at pH 6. Furthermore, whereas Ynt1p is inactivated in the presence of glutamine, nitrite specific activator does not appear to be post-translationally regulated. Therefore, the latter is probably subject only to transcriptional regulation (Machín et al., 2004).

15.2.2 Nitrate Reduction

The first nitrate assimilatory step is the reduction of nitrate to nitrite, operated by nitrate reductase, the peptidic component of which is encoded by *YNR1* (Avila et al., 1995). YNR1p shows high similarity to those of plants and other fungi (Campbell, 1999; Okamoto et al., 1991; Johnstone et al., 1990; Unkles et al., 1992). *H. polymorpha* YNR1p requires various cofactors, including molybdopterinic group, haeme and FAD. Although NADPH is the preferred electron donor, also NADH can serve the purpose (Pignocchi et al., 1998).

Recently, it has been demonstrated in vitro that neither Ynr1p activity nor its stability are impaired by reduced nitrogen sources (ammonium, glutamine), suggesting that this enzyme is not regulated post-translationally (endogenous autoregulation; Navarro et al., 2003).

15.2.3 Nitrite Reduction

Nitrite is reduced to ammonium by nitrite reductase Yni1p, a 116.6 KDa protein encoded by *YNI1* (Brito et al., 1996). Yni1p shows 50% identity with NiR of other fungi. This is a single-copy gene, and no evidence suggests the presence of other isoforms (Siverio, 2002).

As other fungal and bacterial nitrite reductases, *H. polymorpha* Yni1p uses NAD(P)H as electron donors, whereas plants and algae employ ferredoxin (Siegel and Wilkerson, 1989). As typical for nitrite reductases, Yni1p has three prosthetic groups, namely 4Fe-4S center, a siro-haeme group, and a FAD molecule (Campbell and Kinghorn, 1990; Prodouz and Garrett, 1981).

15.3 Nitrate cluster in *H. polymorpha*

In *H. polymorpha,* genes encoding nitrate reductase (*YNR1*, Avila et al., 1995), nitrite reductase (*YNI1,*Brito et al., 1996; Garcìa-Lugo et al., 2000) and nitrate permease (*YNT1*, Pérez et al., 1997) are clustered and independently transcribed (Fig. 15.4; Ávila et al., 1995, 1998; Brito et al., 1996). This nitrate assimilation cluster also contains two regulatory genes (*YNA1,* Ávila et al., 1998; *YNA2,* Ávila et al., 2002) encoding two fungal zinc cluster proteins $Zn(II)_2Cys_6$ (cfr § 13.2.1), involved in nitrate induction mechanisms, as shown by the facts that their null mutants are unable to assimilate nitrate and to induce transcriptional activation of the three structural genes mentioned above.

Garcia-Lugo et al. (2000) showed that in the yeast *H. anomala,* at least two genes involved in nitrate utilisation are also clustered. In the *Aspergilli,* but not in other filamentous ascomycetes (Amaar and Moore, 1998; Johnstone et al., 1990; Kitamoto et al., 1995; Unkles et al., 1992), partial clustering of the nitrate utilisation genes has also been described. Clustering of nitrate assimilatory genes has been recently established in basidiomycetes (Jargeat et al., 2003). The alga *Chlamydomonas reinhardtii* also shows complete clustering of the nitrate assimilatory genes (Quesada et al., 1993, 1998). This phylogenetic distribution and the different gene order within the clusters may suggest that clustering of these genes has occurred independently more than once during evolution. In the *H. polymorpha* nitrate gene cluster the inter-ORF regions, are strikingly short, yielding a coding density as high as 92% (Siverio, 2002).

As mentioned, *YNA1* e *YNA2* encode two zinc cluster proteins, both involved in the induction process (Avila et al., 1998, 2002). Although they belong to the same protein family as the *Asp. nidulans* NirA and *N. crassa* NIT4 proteins (two well studied activators also specifically involved in nitrate assimilation; e. g., Bernreiter

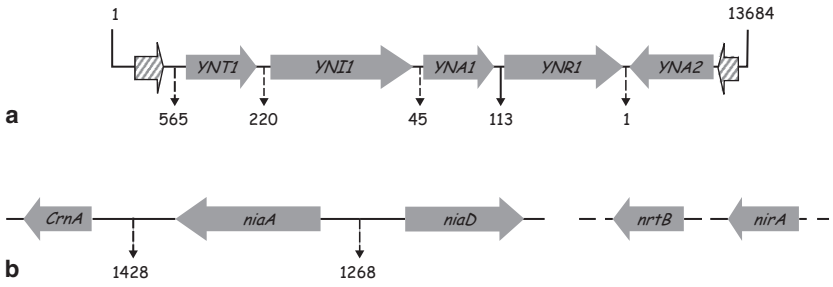

Fig. 15.4 *H. polymorpha* and *Asp. nidulans* nitrate utilization cluster (after Ávila et al.,1998, 2002). Coding regions involved in nitrate assimilation are in light blue (structural genes) or green (regulatory genes). The complete *H. polymorpha* nitrate cluster (A) has five nitrate assimilation genes. ORF and inter-ORF lengths are reported. YNT1, nitrate transporter; YNI1, nitrite reductase; YNA1, Zn(II)2Cys6 transcriptional activator; YNR1, nitrate reductase; YNA2, Zn(II)2Cys6 transcriptional activator; 1, ORF-1; 3, ORF-3. In *A. nidulans* (B) only three genes are clustered (crnA, nitrate transporter; niaA, nitrate reductase; niaD, nitrite reductase)

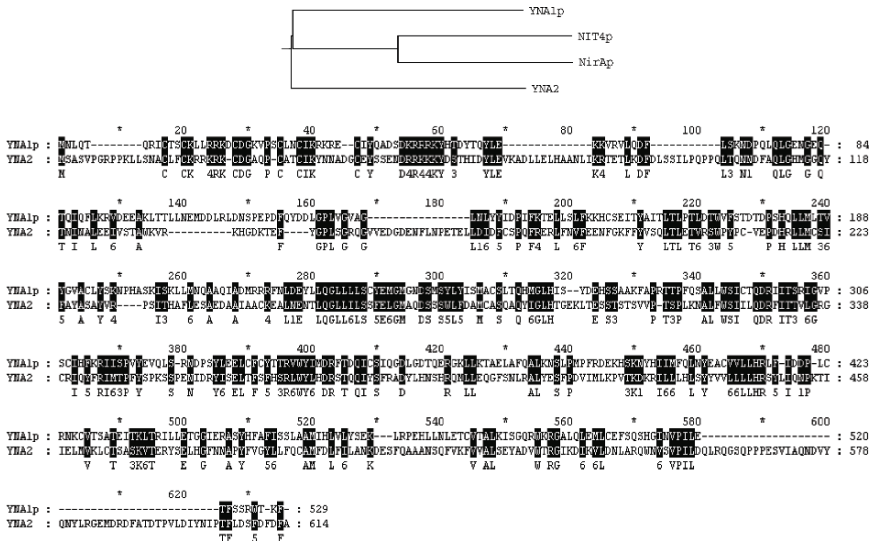

Fig. 15.5 Sequence relations of four nitrate specific trans-activators (above), and alignment of Yna1 and Yna2. Yna1p and Yna2p are from *H. polymorpha;* NIT4p is from *N. crassa*; NirAp is from *Asp. nidulans*

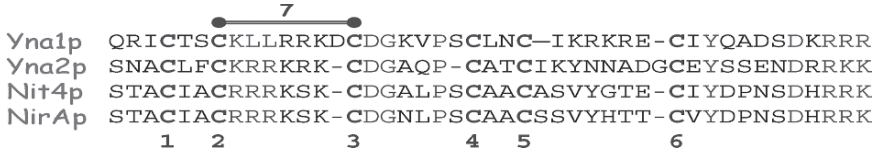

Fig. 15.6 Alignment of zinc cluster region of *trans*-acting factors Yna1 and Yna2 (*H. polymorpha*), Nit4p (*N. crassa)* and NirA (*Asp. nidulans).* This alignment shows the unique Cys-Cys spacer of Yna1, made of seven aminoacid, instead of six, as typical of all other member of this class of fungal transcriptional activators

et al., 2007; Berger et al., 2006; Burger et al., 1991a, b; Feng et al., 1996, 1998; Fu et al., 1995; Marzluf, 1997; Narendja et al., 2002; Strauss et al., 1998; Yuan et al., 1991) they share little similarity with these activators, except in the DNA binding domain (Fig. 15.5).

This similarity is no greater than that found with functionally unrelated proteins belonging to the same family. Yna1p shows a uniquely deviant Zinc cluster motif with seven amino acids, instead of six, between the second and the third cysteines (Avila et al., 1998).

A putative leucine-zipper follows the DNA binding domain of Yna2p. No such motif was found in Yna1p (Avila et al., 1998, 2002). Both, Yna1p and Yna2p are necessary for the expression of the nitrate assimilation genes *YNT1, YNI1* and *YNR1*. Whereas Yna1p appears to be required for *YNA2* induction, Yna2p is not needed for *YNA1* transcriptional activation (Avila et al., 2002). Recent work in our laboratory shows that, in the absence of Yna2p, *YNA1* expression is more sensitive to nitrogen catabolite repression than the wild type (Rossi, 2005) (Fig. 15.6).

15.4 Nitrate Assimilation Control in *H. polymorpha*

As yet, the amount of data available on nitrate assimilation control mechanisms in *H. polymorpha* does not allow to sketch out a model clearly indicating the complex interplay among different elements implicated in this process. However, combining various studies carried out both in Siverio's lab and by us, new important information has recently emerged, especially regarding the two clustered transcriptional activators, namely Yna1p and Yna2p.

As already described, the utilization of secondary nitrogen sources is only allowed in the absence of primary sources, combined to the presence of a secondary nitrogen sources, generally referred to as inducer (Avila et al., 1995; Brito et al., 1996; Pignocchi et al., 1998). Physiologic response elicited by primary sources and leading to repression of genes essential for the assimilation of a secondary sources is generally referred to as *nitrogen metabolite repression,* NMR (a denomination more frequently used in *Aspergillus* and *Neurospora,* and now in *H. polymorpha;* or as *nitrogen catabolic repression,* NCR (a denomination common in *S. cerevisiae;* Cooper, 1982; Cooper and Sumrada, 1983).

We have recently shown, in collaboration with Claudio Scazzocchio (Paris), the existence of a hierarchy in the repressing power of various nitrogen sources with respect to nitrate cluster genes in *H. polymorpha*. Glutamine shows the highest repressive power, followed by glutamate and ammonium. Proline was shown to be a *non-repressing, non-inducing source*, i.e. in regulatory terms, a *neutral* nitrogen source. Therefore, using proline as sole nitrogen source, experiments can be done in a physiological condition generally referred to as de-repression (Rossi, 2005).

Work in our laboratory showed that nitrate cluster structural genes *YNT1,* and *YNI1* and *YNR1* exhibit induction kinetics that are different to those observed for *YNA1* and *YNA2,* the cluster genes with positive regulatory functions. The former reaches high transcriptional levels only after 90–120 induction, while the latter strongly express after 10–20 min induction (Rossi, 2005).

In *H. polymorpha* rapamycin (an antifungal immunosuppressive macrolide; cfr § 13.2.2) blocks NMR, allowing *YNR1* expression even in the presence of glutamine or ammonium (Navarro et al., 2003). This fact suggests the involvement, in *H. polymorpha,* of TOR proteins (*T*arget *o*f *R*apamycin; see Cooper, 2002 and references therein) in post-translational regulation of nitrate assimilatory pathway, similarly to what is known in *S. cerevisiae* for other secondary nitrogen sources (Cooper, 2002 and references therein). This hypothesis is reinforced by the fact that *H. polymorpha* genome shows various ORFs encoding putative proteins which are highly similar to various *S. cerevisiae* factors participating in the TOR mechanism (J. Siverio, personal communication, Fig. 15.7).

Machín et al. (2004); Navarro et al. (2006) showed that, whereas nitrate reductase (Ynr1p) is not involved in the regulation of nitrate utilisation flux, nitrate transporter Ynt1p seems to play a key role in this game. As said, in addition to transcriptional regulation, Ynt1p is subject to post-translational regulation, and is rapidly inactivated in the presence of reduced nitrogen sources such as glutamine.

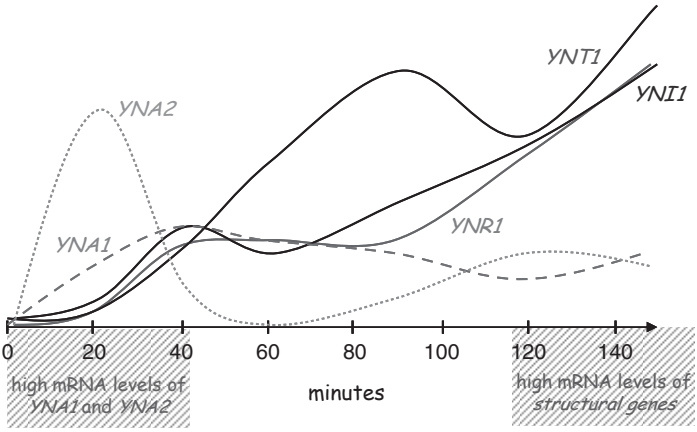

Fig. 15.7 Summary of typical mRNA induction dynamics of the nitrate assimilation genes in *H. polymorpha* after shift from proline to nitrate

A little puzzling is the presence of two homologous, albeit different, transcriptional activators acting upon the genes of the nitrate cluster, and both essential for their transcriptional activation (Yna1p e Yna2p; Brito et al., 1996; Ávila et al., 2002) – a pattern which differ from those characteristic of other fungal nitrate assimilatory pathways (*Aspergillus, Neurospora*, etc.). Work in our laboratories suggest that Yna1 positively influences also *YNA2*, whereas Yna2 does not act over *YNA1* expression, suggesting that auto-induction of *YNA1* is a possible inducing mechanism, with either Yna1p directly binding to *YNA1* promoter, or inducing a second gene, whose product could bind to *YNA1* promoter. Of course, other models are possible, including the involvement of an AreA-like GATA factor (hypothesised, but yet to be discovered) sufficient to induce *YNA1* (Fig. 15.8).

Our recent work has, however, showed that *YNA2* plays a central role in chromatin remodelling within *YNT1* and *YNI1* promoter regions, a task implicated by GATA factor AreA in *Asp. nidulans.*

It is largely recognised that chromatin remodelling plays an important role in eukaryotic transcriptional regulation. Disruption of nucleosomal structure accompanies the activation of many genes, and *H. polymorpha* nitrate gene cluster is no exception to that. In non-expressing conditions, *YNI1* and *YNT1* show highly structured chromatin organisation and well positioned nucleosomes. These structures undergo profound remodelling during gene activation (i.e. during nitrate growth; Rossi, 2005). Parallel analyses with *yna1 Δyna2 Δ*, showed that Yna2 is directly involved in those remodelling processes, whereas *YNA1* may only play an in direct role by activating *YNA2.*

Information on NMR mechanisms acting upon nitrate assimilative genes in *H. polymorpha* was obtained in our laboratory through the isolation and characterisation of Nmr mutants (Serrani et al., 2001; Rossi et al., 2005) (Fig. 15.9).

Study of mutants FM-32B3 (*nmr1-1*), FM-49A1 (*nmr2-1*) e FM-101B (*nmr4-1*) contributed to validate the idea that also in *H. polymorpha* repression mechanisms

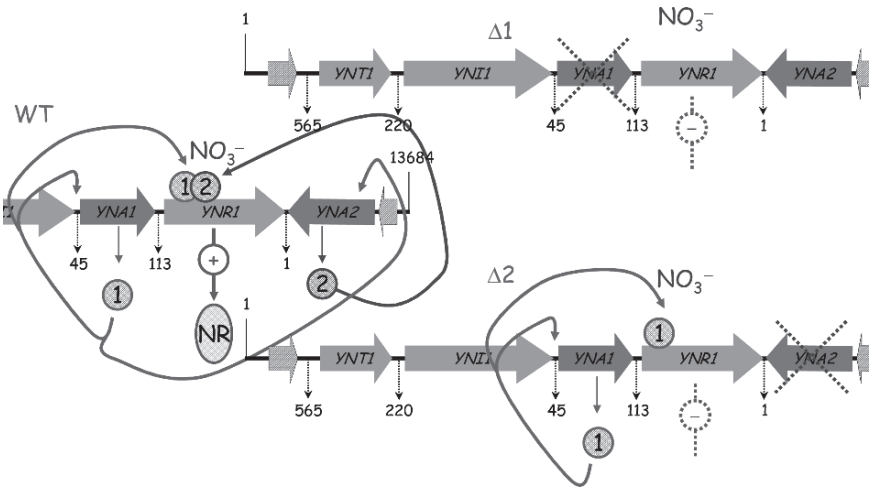

Fig. 15.8 Model showing possible role of Yna1 activator in the presence of nitrate (see text for details). Alternatively, a GATA factor may suffice to activate *YNA1*

are mediated by many interacting factors (Brito et al., 1996; Pignocchi et al., 1998; Serrani et al., 2001; Rossi et al., 2005). These factors are believed also to participate in nitrogen metabolite repression of other metabolic pathways, all involved in the utilisation of secondary nitrogen sources such as arginine, methylamine, urea and asparagine (Serrani et al., 2001; Rossi et al., 2005).

In particular, mutations in the *NMR1 locus* bring about de-repression of nitrate assimilation in the presence of glutamate, but not of glutamine suggesting that a glutamine-depending signalling circuit may coexist with a glutamate-depending one (Serrani et al., 2001). The presence of two regulatory circuits, a glutamate dependent one, and a glutamine dependent one, has been recently proposed also for *Asp. nidulans* (Margelis et al., 2001; Morozov et al., 2001; Mihlan et al., 2003) and constitutes a novelty.

The physiological and biochemical characterization of *nmr2-1* suggest that *NMR2,* as well as *NMR1,* is part of a negative regulatory response to reduced nitrogen compounds. However, *nmr2-1* causes a more general de-regulated phenotype, affecting nitrogen metabolite repression of NR in the presence of both, glutamine and glutamate and all other nitrogen source tested.

All together our data suggest that *NMR2* and *NMR1* are involved at different levels of the NMR regulative circuit. In particular, a reasonable hypothesis is that *NMR2* and *NMR1* have a role similar to the one of the negatively acting proteins *Asp. nidulans* NmrA, *N. crassa NMR1* or *S. cerevisiae* Ure2p, which inhibits AreA, NIT2 and Gln3p, respectively. Mutations in proteins involved in TOR signalling cascade acting on nitrogen metabolism could also be argued (Cooper, 2002 and references therein, Fig. 15.10).

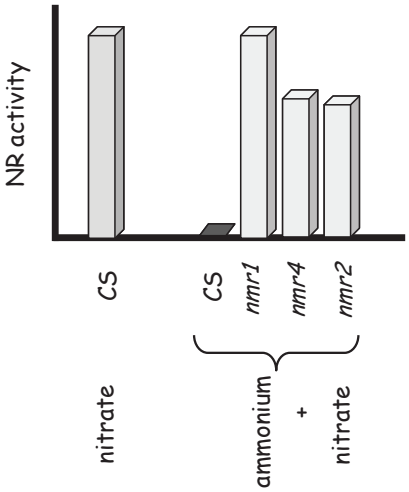

Fig. 15.9 Summary of *H. polymorpha nmr* mutants characterised so far (CS, control strain)

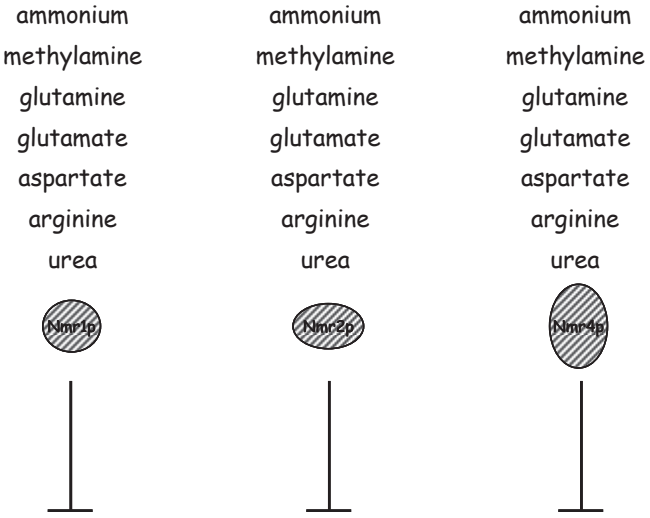

Fig. 15.10 Summary of Nmr1p, Nmr2p, Nmr4p roles. All three proteins are involved in *H. polymorpha* nitrogen metabolite repression of nitrate assimilation elicited by red, but not by green compound. Since Nmr2p is involved in Nmr exerted by all nitrogen compounds tested, it has been suggested that this protein may act downstream of Nmr1 and Nmr4p. Certain phenotypic traits of Nmr4 make it possible for this protein to act as an ammonium sensor. It has been suggested that a glutamine dependent circuit exists, separated from an ammonium dependent circuit

The characterisation of the *nmr4-1* mutant suggests that *NMR4* could act as a sensor also participating in ammonium assimilation. Further characterisation of this mutant, currently under way, may help to elucidate its role in nitrogen metabolite repression of *H. polymorpha*.

15.5 Conclusions

Interest in *H. polymorpha* has been steadily growing in recent years, both biologically and for commercial application. This yeast plays a leading role in peroxisome and heterologous protein expressions. As shown in this chapter, *H. polymorpha* is proving itself also an excellent model for nitrate assimilation studies. In spite of the small community involved in these studies, our understanding on *H. polymorpha* nitrate utilisation has deepened, adding significant molecular details to the previous portrait. In particular, recent exciting data concern transport studies, down-regulation of Ynt1p by ubiquitinylation processes and the role of Yna1p and Yna2p in the regulatory mechanisms controlling nitrate metabolism. As far as gene arrangement and functional organisation are concerned, it is noteworthy that *H. polymorpha* data, placed in relation to those of other well-established fungal models, have also a bearing in the evolutionary understanding of fungal nitrate assimilation. It is hoped that more research will soon enlarge this increasingly complex picture.

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