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Unique status of *NIA2* in nitrate assimilation

NIA2 expression is promoted by HY5/HYH and inhibited by PIF4

Else Müller Jonassen, Bjørnar A.A. Sandsmark and Cathrine Lillo*
Centre for Organelle Research; Faculty of Science and Technology; University of Stavanger; Stavanger, Norway

Light perceived by phytochromes will induce genes of nitrogen assimilation, however, transducing components in the signaling cascades to these genes are hardly known. Recently the bZIP transcription factors HY5 (LONG HYPOCOTYL5) and HYH (HOMOLOG OF HY5) were identified as positive regulators in light activation of *NIA2 (nitrate reductase 2)*. The bHLH transcription factor PIF4 (PHYTOCHROME INTERACTING FACTOR 4) was revealed as an inhibitor of *NIA2* expression. In contrast to *NIA2*, expression of other genes of nitrogen assimilation, *NRT1.1 (dual-affinity nitrate transporter 1.1), NIA1 (nitrate reductase 1), NIR (nitrite reductase), GLN2 (glutamine synthetase 2)* and *GLU1 (glutamate synthase 1)* were not promoted by HY5/HYH or inhibited by PIF4. *NIA2* as the outstanding gene of nitrate assimilation regarding HY5/HYH and PIFs may have evolved in connection with the cytosolic leaf localization of nitrate reductase, and adverse effects of the products, nitrite, nitric oxide and active oxygen species formed by the enzyme.

Key words: bHLH, bZIP, HY5, nitrogen assimilation, nitrate reductase, phytochrome, PIF4

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*Correspondence to:
Cathrine Lillo; Email: cathrine.lillo@uis.no

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In seedlings, light perceived by phytochromes induces activation of genes promoting photomorphogenesis as strikingly characterized by short hypocotyls, cotyledon expansion and greening. Various downstream components of phytochromes are involved, and the bZIP transcription factor HY5 (LONG HYPOCOTYL5) is crucial in many of the signaling cascades promoting photomorphogenesis.[1,2] A group of bHLH transcription factors called PIFs (phytchrome interacting factors) are also downstream factors of phytochromes, and may promote or counteract responses to light.[3-5] Some genes forming a functional cluster are regulated by a common downstream factor as found for example in a subset of the flavonoid pathway genes activated by HY5 binding to their promoters. Furthermore, these flavonoid pathway genes are also activated by binding of a PIF (PIF3) to their promoters.[6] Light perceived by phytochromes will lead to rapid degradation of PIFs[1] thereby influencing PIF-dependent processes. Effects of different PIFs can be additive;[7,8] single *pif* mutations had only small effects, whereas a constitutive photomorphogenetic phenotype (even in darkness) was found for seedlings of the quadruple *pif1pif3pif4pif5* mutant.[8] The full picture of phytochrome signaling is far from clear, and indeed very complex since components can act both as inhibitors and enhancers, depending on environment, tissue and process in question. Also a range of different phytochromes are present (five in Arabidopsis) which add complexity by homodimerizing and heterodimerizing.[9] Although phytochrome signaling to genes of photosynthesis and flavonoid metabolism has been more intensively studied, light absorbed by phytochromes has also long been known to induce activation of genes in the nitrogen assimilation pathway in various plant species.[10-12] However, downstream components of phytochromes in the signaling pathways to nitrogen metabolism have hardly been investigated.[1,13]

We examined the involvement of phytochromes and putative downstream factors in nitrogen assimilation by comparing various loss-off-function mutants.[14,15] HY5

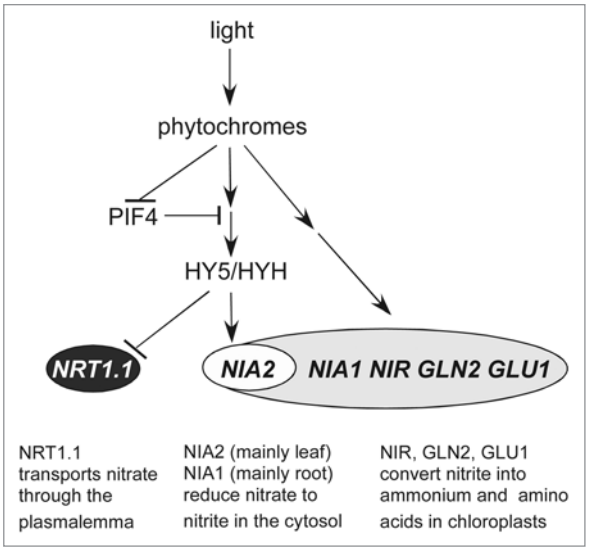

Figure 1. Schematic diagram summarizing positive and negative light effects on genes in the nitrate assimilation pathway of Arabidopsis seedlings. Continuous light (red, far-red, white) induced *NIA2* and other genes involved in nitrate assimilation, whereas *NRT1.1* was weakly inhibited by light. Light effects on *NIA2* and *NRT1.1* were enhanced by the presence of *HY5/HYH*. PIF4 was identified as an inhibitor of *NIA2* expression. Our data are compatible with PIF4 acting through the HY5/HYH pathway because *NIA2* and *NRT1.1* were oppositely influenced in the *pif4* mutant, and other genes of nitrate assimilation were not influenced. However, the available data do not determine if PIF4 rather act through a separate signaling cascade (not including HY5/HYH) to inhibit *NIA2*.

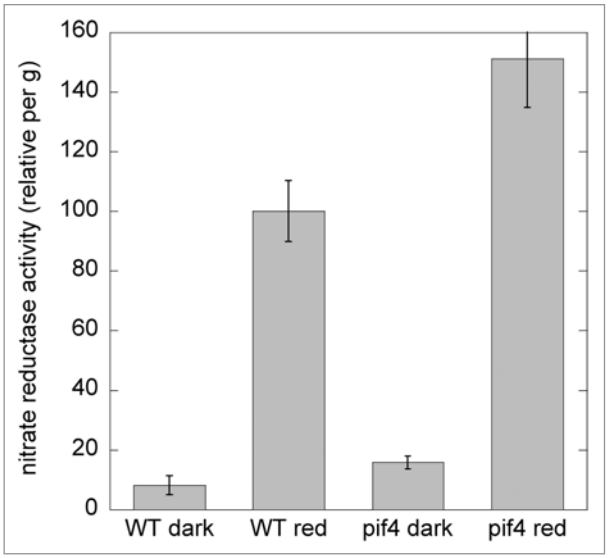

Figure 2. Nitrate reductase activity is enhanced in *pif4* seedlings grown in continuous red light (4 μmol $m^{-2}s^{-1}$) for six days compared with wild type (WT). Experimental conditions were as previously described.[14,15] Data presented are means from four independent experiments. SE is given. NR activity was significantly higher in the *pif4* mutant compared with wild type in red light ($p < 0.05$).

and HYH (HOMOLOG OF HY5) were shown to be positive modulators of far-red and red-light-signaling to *NIA2*. *NIA2* is one of the two nitrate reductase genes in Arabidopsis, and is the gene dominantly expressed in green leaves. Impairment of *NIA2* expression in the *hy5 hyh* double mutant[15] underpinned our previously findings that presence of *HY5/HYH* was a prerequisite for high nitrate reductase (NR) activity in seedlings grown in continuous red or far-red light.[14] HY5/HYH as possible enhancers also of other genes in the nitrate assimilation pathway were tested.[15] The genes examined were *NRT1.1*, which is established as a gene important for nitrate uptake in Arabidopsis,[16] *NIA1* (root-important *NIA*), and genes encoding enzymes for the subsequent steps in nitrate assimilation, i.e., *NIR* (*nitrite reductase*), *GLN2* (*glutamine synthetase 2*), and *GLU1* (*glutamate synthase 1*) (**Fig. 1**). In contrast to *NIA2*, the gene for nitrate uptake was negatively affected, and expression levels of the other genes of the nitrogen assimilation pathway were not influenced by presence of *HY5/HYH*.[15] This is consistent with the genome wide analysis of HY5 binding by Lee et al.[2] using a chromatin immunoprecipitation procedure, which revealed putative binding sites for HY5 in the *NIA2* and *NRT1.1* promoters, but not other genes depicted in **Figure 1** (Suppl. material in Lee et al.). We also tested NR activity in single *pif* mutants (*pif1*, *pif3* and *pif4*),[5,7] which showed that NR activity was increased by 50% in the *pif4* mutant in comparison with wild type in red-light-grown seedlings (**Fig. 2**). The positive effect on NR activity by the *pif4* mutation was confirmed also in rosette leaves exposed to 72 h of darkness followed by 4 h red light. Interestingly, only *NIA2* transcript level was clearly increased in *pif4*; expression of *NRT1.1* was decreased, and other genes of nitrate assimilation were not consistently influenced (Sandsmark, unpublished). Recently, microarrays were used to investigate gene expression in a *pif1pif3pif4pif5* quadruple mutant by Shin and coworkers.[8] *NIA2* was the only gene of nitrogen assimilation revealing enhanced expression in the quadruple mutant (Suppl. material in Shin et al.),[8] which confirms the exceptional regulation of the *NIA2* gene as opposed to other genes of nitrate assimilation regarding PIFs. This places PIF4 as an inhibitor of light-induced *NIA2* expression in the signaling pathway (**Fig. 1**). It is intriguing that *NIA2* expression, but not other genes of nitrate assimilation is activated by HY5/HYH and inhibited by PIF4. The reason for this is not known, but possibly the special localization of NR to the cytosol as opposed to further steps taking place in the chloroplasts, and the fact that NR produces products that can be toxic (nitrite, nitric oxide, reactive oxygen species)[17] may have promoted evolution of the

complex fine-tuning of *NIA2* expression different from the other genes of nitrate assimilation.

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