ARTICLE ADDENDUM

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Phosphorylation by MPK6

A conserved transcriptional modification mediates nitrate reductase activation and NO production?

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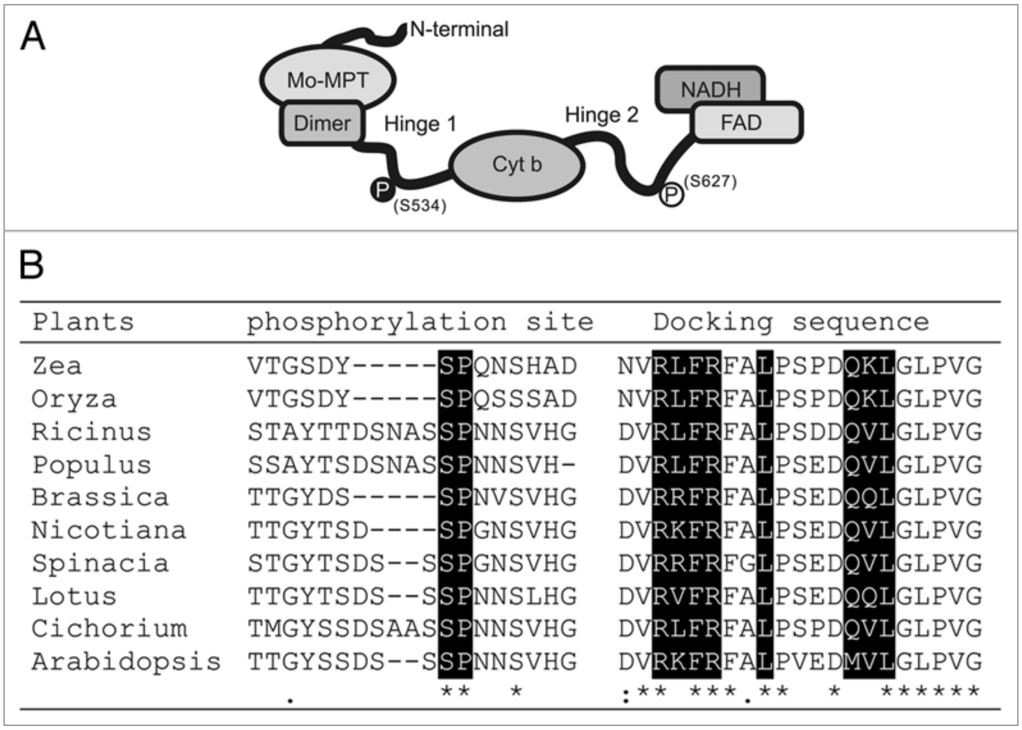
Nitrate reductase is a central enzyme of nitrogen assimilation in plants. In a recent work, we have revealed MPK6 could phosphorylate Arabidopsis NIA2 at the serine 627 in hinge 2 region, this phosphorylation may represent a rapid activation mechnism when plant need excessive nitrate reduction. Interestingly, all eukaryotic NRs have conserved docking sequence in their FAD domains, and many plant NR proteins have the conserved MAPK phosphorylation site. Those indicated that phosphorylation of NR protein by MAP kinase cascade may be conserved in different species. We noticed that the phosphorylation of S627 residue by MPK6 have a specially influence on the NO generation. Although no homology of mammalian NOS has been identified in high plants, NR may still share a similar regulation mechanism with mammalian NOS.

Nitrogen assimilation is a vital process controlling plant growth and development. In high plants, nitrate is the major nitrogen source, after taken up into plant cells, it must be reduced to ammonia for further usage. As the first enzyme in nitrate reduction pathway, the nitrate reductase (NR, NIA) is critical for regulation of the nitrogen assimilation.[1] It is well documented that the amount and activity of NR is tightly controlled at transcriptional and pos-transcriptional levels by nitrate, light and CO_2 levels, circadian rhythms, nitrogen and carbon metabolites, phytohomones, etc. Post-translational mechanisms could reversibly modulate NR activity within minutes and permit quick responses to environmental and cellular metabolism changes, which is the dominant regulation mechanism of NR activity.

Generally, plant NR protein contains three catalytic domains: Mo-molybdopterin (Mo-MPT) and interface domain, cytochrome b (Cyt b) domain, and FAD and NADH domain. Hinge 1 and hinge 2 regions are localized between those domains and joined them together (**Fig. 1A**).[1] Emerging evidences have indicated that the phosphorylation of hinge 1 and hinge 2 regions have dominant influences on the NR activity. For example, phosphorylation of hinge 1 region at serine residue in Arabidopsis (S534), spinach (S543) or tobacco (S521) inhibited NR activities.[2-4] The phosphorylation may coresponding to rapidly inactivate NR in response to severa1 signals, including dark, decrease in CO_2 levels or increase in cytosolic pH.[1,3] On the contrary, phosphorylation at hinge 2 region showed a positive effect on NR activity, site-directed mutagenesis of the serine (S627) to aspartic acid, which mimic the phosphyorylation form of NIA2, caused the increase of NR activities about 2.5-fold.[5] Interestingly, application of exgenous reactive oxygen species (ROS),[5] or accumulation endogenous ROS in some condition, cause the rapid activation of NR via phosphorylation at this site. For example, during light-to-dark transitions, release of single oxygen is coupled with activation of NR.[6,7] Application of exogenous salicylic acid or accumulation of endogenous salicylic acid in *rcd1*, induced the ROS generation, also could activate MPK6,[8] and increase total NR



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Plants       phosphorylation site   Docking sequence

Zea          VTGSDY-----SPQNSHAD   NVRLFRFALPSPDQKLGLPVG
Oryza        VTGSDY-----SPQSSSAD   NVRLFRFALPSPDQKLGLPVG
Ricinus      STAYTTDSNASSPNNSVHG   DVRLFRFALPSDDQVLGLPVG
Populus      SSAYTSDSNASSPNNSVH-   DVRLFRFALPSEDQVLGLPVG
Brassica     TTGYDS-----SPNVSVHG   DVRRFRFALPSEDQQLGLPVG
Nicotiana    TTGYTSD----SPGNSVHG   DVRKFRFALPSEDQVLGLPVG
Spinacia     STGYTSDS--SSPGNSVHG   DVRRFRFGLPSEDQVLGLPVG
Lotus        TTGYTSDS--SSPNNSLHG   DVRVFRFALPSEDQQLGLPVG
Cichorium    TMGYSSDSAASSPNNSVHG   DVRLFRFALPSPDQVLGLPVG
Arabidopsis  TTGYSSDS--SSPNNSVHG   DVRKFRFALPVEDMVLGLPVG
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Figure 1. MAPK phosphorylation site and docking sequence are highly conserved in NR proteins. (A) Sequence model of the Arabidopsis NIA2 protein, phosphorylation sites S534 and S627 were marked. (B) Amino acid sequence comparison around the putative MAPK phosphorylation site and docking sequence (high lighted) of NR in high plants.

activity.[9] Phosphorylation at S627 of NR by MPK6 may represent a rapid activation mechnism when plant need excessive nitrate reduction.

Interestingly, phosphorylation by MPK6 might be a conserved modification mechanism of NR. All eukaryotic NRs have conserved docking sequence in their FAD domain, which may be necessary for recognition of MAP kinase (**Fig. 1B**).[10] NR proteins in some dicots and monocots plant species, including Barssica, tobacco, Lotus, rice, Maize, Cichorium and spinach, have the conserved MAPK phosphorylation site (SP residue, high lighted in **Fig. 1B**) at hinge 2 region. It has been reported that the hinge 2 evolved fastest in all domains in NR protein.[11] High conservation of MAPK recognition and phosphorylation site in this region indicates the importance of the post-transcriptional regulation of NR activation, and the modulation at hinge 2 region by MAP kinase may be responsible for the accurate control of NR activity under certain internal or environmental conditions.

MAP kinase cascades are conserved signal transduction cascade that transduce extracellular stimuli into intracellular responses in yeast and animal cells. In plant, MAP kinase also modulates various biological progresses. MPK6, one of well-characterized MAPK in plant, mediated innate immunity,[12,13] ethylene and jasminate signaling,[14-17] abiotic stresses,[18] leaf senescence,[19] stomotal,[20] anther,[21] ovule[22] and root development.[23] The diverse function of MPK6 suggested the central role of MAPK cascade in intracellular signaling network. Our work indicated the conserved signaling cascades also involved in lateral root development, by modulation of NR phosphorylation and NO synthesis.

The primary function of NR to is reduce nitrate to nitrite, somehow, it also catalyzes the nitric oxide (NO) production.[24,25] NO generation induced by auxins, abscisic acid or stresses are dependent on NR activity.[26-28] Recent works have addressed the relationship between mitogen-activated protein kinase (MAPK) cascades and NO generation in tobacco and Arabidopsis.[5,29] So far, details about the biochemical progresses and regulatory mechanisms of NR dependent NO generation are still largely unknown. However, phosphorylation of NIA2 also increased the NO generation dramatically in transgenic plants, but this modulation have different effects on NR activity and NO generation.[5] For example, overexpression of *NIA2* induced the NR activity more than eight-fold, but it only stimulated the NO generation slightly. On the contrary, application of exogenous H_2O_2 has a stronger effect on NO generation than NR activity in both wild-type and *NIA2*^{WT} transgenic plants, but not in *NIA2*^{D} and *NIA2*^{A} plants. A possibility is that the phosphorylation of S627 by MPK6 have a specially influence on the NO generation activity of NIA2. In mammalian, a serine/threonine protein kinase Akt (protein kinase B) can directly phosphorylate endothelial nitric oxide synthase (eNOS) on serine 1179 and activate the enzyme, leading to NO production.[30,31] According to this, *mpk6* mutant showed lower NO accumulation and enhanced lateral root development under the application of NO donor sodium nitroprusside (SNP) or H_2O_2. Although no homology of mammalian NOS has been identified in high plants,[32] NR may still share a similar regulation mechanism with mammalian NOS.

Acknowledgements

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