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Arabidopsis cytosolic glutamine synthetase AtGLN1;1 is a potential substrate of AtCRK3 involved in leaf senescence

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Abstract

While considerable progress has been achieved in plant CDPK studies in the past decade, there is relatively no information about the potential substrates of CRKs. In this report, a yeast two-hybrid screen was performed with truncated form of AtCRK3 as bait to identify its interacting proteins in an effort to dissect its physiological roles. One gene encoding cytosolic glutamine synthetase *AtGLN1;1* was isolated. Further analyses indicated that AtGLN1;1 could interact specifically with AtCRK3 and the kinase domain of AtCRK3 and the catalytic domain of AtGLN1;1 were responsible for such interaction, respectively. Furthermore, in vitro and in vivo co-immunoprecipitation results strongly supported that they could physically interact with each other. Phosphorylation assays revealed that AtGLN1;1 could be specifically phosphorylated by AtCRK3 in vitro. All the results demonstrate that AtGLN1;1 may be a substrate of AtCRK3. In addition, both *AtGLN1;1* and *AtCRK3* could be induced by natural or artificially induced leaf senescence, implying that such interaction may be involved in the regulation of nitrogen remobilization during leaf senescence.

Keywords: *Arabidopsis*; CRK; Glutamine synthetase; Phosphorylation; Senescence; Substrate

Calcium signaling is one of the best documented pathways in plants. It has been demonstrated to be operative in a series of biological processes from cell division to plant responses to a wide range of stimuli including hormones, light, pathogen elicitors, and abiotic stresses [1–4]. The roles of Ca^{2+} are mediated by a group of Ca^{2+}-binding proteins including CaM (calmodulin) and CDPKs (Ca^{2+}-dependent protein kinases). CDPKs are serine-threonine protein kinases that have a conserved modular structure of four domains: an N-terminal variable domain, a protein kinase domain, an auto-inhibitory junction domain, and a C-terminal calmodulin-like domain containing EF-hand Ca^{2+}-binding motifs. CDPKs are activated upon binding calcium to their calmodulin-like domain, which makes them effective switches for the transduction of calcium signals in plant cells [2,3].

CDPKs make up a super-family of protein kinases in plants. In *Arabidopsis*, CDPKs form one of the largest Ca^{2+}-binding protein families, with 34 unique CDPK genes plus 8 CDPK-related kinases (CRKs) [3]. Although the structures of CRKs and CDPKs are similar, CRKs are characterized by a regulatory domain that has high sequence similarity to the CaM-LD of CDPKs, but with EF-hands that seem to have degenerated and are predicted to no longer bind Ca^{2+}. Biochemical data obtained from DcCRK, ZmMCK1, and AtCRK3 confirmed that the kinase activity of these proteins did not require calcium [5–9]. However, there is evidence showing that some CRK isoform could be activated by exogenous Ca^{2+}/calmodulin [10].

Substrate specificity, calcium-activation threshold, and temporal cyto-localization are thought to be mechanisms by which CDPKs specifically translate calcium fluxes into physiological responses [2]. Although there has been considerable progress in plant CDPK studies from identifying

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the new isoforms and cloning corresponding genes, only a few of CDPK isoforms have clearly been assigned functions [2,4]. A wide variety of potential substrates have been described in in vitro biochemical analysis, which supports the view that conventional CDPKs are multifunctional kinases that are involved in the regulation of diverse aspects of cellular function [2]. Very few of these proteins, however, have been identified as bona fide substrates of individual CDPKs [1,3]. So major challenges for the future would be to identify functions for individual CDPK isoform and to provide a picture of how CDPK signaling is used in plant development and physiology. In contrast to CDPKs, there is relatively no information about the potential substrates of the CRKs [4]. Therefore, it is still unclear to what extent these different kinases have unique or overlapping sets of substrates.

Nitrogen assimilation is a vital process controlling plant growth and development [11]. In plants, the major route involved in ammonium assimilation is the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle. GS catalyses the first step in the conversion of inorganic nitrogen (ammonium) into an organic form (glutamine). The GS enzymes occur as distinct isoforms: a cytosolic form (GS1) and a plastidic form (GS2). The genes encoding GS1 have been described as a small multigene family while the gene encoding GS2 is a single gene in higher plants. Chloroplastic GS2 has been proposed to function in primary assimilation of ammonia reduced from nitrate and the reassimilation of photorespiratory ammonia. As cytosolic GS, GS1 has been proposed to function in root nitrogen assimilation or intercellular nitrogen transport. The isozymes of GS1, which show organ- and cell-specific patterns of expression, are developmentally and environmentally regulated [12]. In *Arabidopsis*, five putative genes for GS1, *AtGLN1*;*1*, *AtGLN1*;*2*, *AtGLN1*;*3*, *AtGLN1*;*4*, and *AtGLN1*;*5*, are encoded in the genome [13]. However, the exact functional roles and physiological diversities of the individual GS1 isozymes in *Arabidopsis* have not been well characterized [13,14]. Recent work suggests that both GS1 and GS2 were regulated not only transcriptionally but also post-translationally [15]. Oxidative modifications of GS1 resulted in an inactive enzyme more susceptible to degradation than non-oxidized GS1 [15]. Phosphorylation of GS1 and GS2 from different plant species also put forward another interesting regulation mechanism, but neither function of these processes nor the requisite kinase(s) was well explored [16–20].

In a previous work, we have biochemically characterized an *Arabidopsis* CDPK-related protein kinase, AtCRK3 [6]. In this report, we attempted to address biological functions and related signaling pathway of AtCRK3 by identifying substrates or interactive regulatory proteins. Here we show evidences that AtCRK3 could specifically interact with and phosphorylate a cytosolic glutamine synthetase, AtGLN1;1, and their possible role in the control of nitrogen remobilization during leaf senescence is discussed.

Materials and methods

Plant material and growth conditions. Arabidopsis ecotype Columbia (Col-0) was used for all experiments. Plants were cultured in a growth chamber controlled at 22 °C with 60% relative humidity under 16 h light and 8 h dark cycles. Leaf samples representing various progressive senescence stages were harvested at different time points, immediately frozen in liquid nitrogen and stored at −80 °C until analyzed. Senescent leaf samples were divided into three groups: S1, S2, and S3 leaves with 25%, 50%, and 75% of the leaf area yellowed, respectively. For senescence induction, leaves from 3- to 4-week-old plants were excised and incubated in permanent darkness on wet filter paper for up to 7 days at ambient temperature. All experiments were performed with the fourth rosette leaves. To avoid changes in expression that might be due to circadian regulation, samples were always harvested 3 h after the beginning of illumination.

Plasmid construction. Molecular biological experiments were carried out according to the standard protocols [21]. For the interaction test, different deletions of the genes were cloned into yeast two-hybrid vectors and in frame either fused with the GAL4 AD or BD domains. For bait constructs, full length open reading frame form of AtCRK3 (1–595), N-terminal variable region plus the kinase domain AtCRK3P1 (1–401), N-terminal variable domain AtCRK3P2 (1–130), kinase domain AtCRK3P3 (131–401), kinase domain minus the ATP binding site AtCRK3P4 (175–401), and N-terminal variable region plus the kinase domain AtCRK1P1 (1–404) were PCR amplified and cloned into the *BamH1* site of vector pGBKT7. For prey constructs, the full length open reading frame form of AtGLN1;1 (1–356), beta-Grasp domain AtGLNP1 (1–102), and catalytic domain AtGLNP2 (103–356) were PCR amplified and cloned into the *SalI* site of vector pGADT7.

For the expression of the recombinant protein, *AtCRK3* and *AtCRK3P1* were amplified and cloned into *BamH1* site of plasmid pFastBacHTb for expression in sf9 insect cells. The forward primer was designed to add codons for an N-terminal c-myc epitope (5′-CGG GATCCATGGAGGAGCAGAAGCTGATCTCAGAGGAGGACCTG ATGGGGCAATGTTACGGGAAGG-3′). *AtGLN1*;*1* and *AtGLNP2* were amplified and cloned into the *SalI* site of pET28a for expression in *Escherichia coli* cells. The reverse primer was designed to add codons for a C-terminal HA (hemagglutinin) epitope (5′-GCGTCGACAGCGTAAT CTGGTACGTCGTAAGGATTCCAGAGGATTGTAGTC-3′).

For in vivo immunoprecipitation, the full length form of *AtCRK3* was first cloned into vector pMENCHU with HA epitope. In contrast the full length form of *AtGLN1*;*1* was cloned into vector pGIGI with c-myc epitope. The expression cassette of the intron-tagged c-myc epitope-labeling vector pGIGI-*AtGLN1*;*1* was then inserted as a blunt-ended *NotI* fragment into filled-in *EcoRI-BamHI* sites of the binary vector pPCV002 to yield pPCV002-GIGI-*AtGLN1*;*1*. In addition, the expression cassette of the intron-tagged HA epitope-labeling vector pMENCHU-*AtCRK3* was cloned as a blunt-ended *NotI* fragment into filled-in *EcoRI-SacI* sites of the binary vector pPCV812 to yield pPCV812-MENCHU-*AtCRK3*. All insert DNAs were confirmed by DNA sequencing.

Yeast two-hybrid screen and interaction tests. The GAL4-based Matchmaker Yeast Two-hybrid System III (Clontech) was used to screen for proteins interacting with AtCRK3 according to the manufacturer's instructions. The N-terminal region containing kinase domain AtCRK3P1 (1–402) was fused in frame with the DNA-binding domain of vector pGBKT7 using *BamH1* restriction sites to get the bait construct. The construct was then tested for auto-activation in yeast strains AH109 and Y187. Prey constructs were expressed as GAL4 activation domain fusion proteins from plasmid pACT2 as prey library, which was kindly obtained from the *Arabidopsis* Biological Resource Center (ABRC, Ohio State University, Columbus). For the interaction screen, AH109 strain contains the bait construct was subsequently transformed with the library plasmids using the PEG/LiAC/ssDNA method. Then the transformed diploids were spread on SD-Leu/-Trp/-His/-Ade plates (supplemented with 10 mM 3-AT). Clones that grew on these selective plates within 4 days were picked and then β-galactosidase filter lift assay was performed. Clones that