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

Rong-Jun Li [a], Wei Hua [b], Ying-Tang Lu [a,*]

[a] *Key Laboratory of MOE for Plant Developmental Biology, College of Life Sciences, Wuhan University, Wuhan 430072, China*
[b] *Institute of Oil Crops Research, Chinese Academy of Agriculture Sciences, Wuhan 430062, China*

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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

* Corresponding author. Fax: +86 27 68756380.
E-mail address: yingtlu@whu.edu.cn (Y.-T. Lu).

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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

developed a strong blue color after 2 h were analyzed further by PCR using vector primers to amplify the inserts. The amplified products were then sequenced. Deletion derivatives of the identified genes for putative interaction partners were cloned into pGADT7 and then transformed into Y187 to verify the interactions. All were routinely tested for auto-activation.

For interaction specificity and interaction domains analysis, pairwise co-transformants were selected on SD plates minus Trp and Leu, then transferred to be selected on SD-Leu/-Trp/-His/-Ade plates with 10 mM 3-aminotriazole, and be tested for β-galactosidase activity by filter lift assays.

RNA extraction and Northern-blot analysis. Total RNA was isolated with TRIZOL® as described by manufacturer (GIBCO). Northern blotting was carried out as previously described [22]. Briefly, 20 μg total RNA was separated on a 1.5% formaldehyde agarose gel and blotted onto a nylon membrane (NYTRAN®). The membrane was incubated with the ^{32}P-labeled probe made from 3′-untranslated region of *AtCRK3* and *AtGLN1;1* at 65 °C overnight and washed under high stringency conditions. For probe preparation, the cDNAs for 3′-untranslated regions of *AtCRK3* and *AtGLN1;1* were cloned into the vector pBluescript II SK (−) for the probes using the plasmid above as a template by random primer extension as described previously [22].

Purification of recombinant proteins and in vitro protein binding assays. For expression of AtCRK3 and AtCRK3P1 in insect sf9 cells, all experiments were performed as described previously [6]. AtGLN1;1 and AtGLNP1 proteins were expressed in *E. coli* strain BL21 (DE3). Overnight cultures were diluted 100-fold and grown for 3 h at 37 °C. Subsequently, isopropylthio-β-galactoside was added and growth continued for 2 h at 21 °C. Soluble 6XHis fusion proteins were extracted and purified using Ni-NTA superflow (Qiagen, Valencia, CA) following the manufacturer's recommendation. After the elutes were desalted and equilibrated in 25 mM Tris–HCl, pH 7.5, the purity was checked by both 10% SDS–polyacrylamide gel electrophoresis. Soluble protein content was determined by the Bradford method using bovine serum albumin as a standard.

The in vitro binding experiments were performed by mixing purified protein, supernatant lysate, appropriate antibody, and protein G Sepharose as indicated in 200 μl binding buffer (25 mM Tris–HCl, pH 7.5, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1× protease cocktail mix, and 0.01% Triton X-100 with or without 10 mM $MgCl_2$/100 μM ATP). The reaction mix was incubated for 4 h at 4 °C with gentle agitation. The Sepharose beads were collected and washed five times in 1 ml of 50 mM Tris–HCl buffer containing 300 mM NaCl. After the final wash, the beads were resuspended in 50 μl of 2.5× SDS–PAGE sample buffers and boiled for 5 min. Eluted proteins were subjected to SDS–PAGE and transferred onto polyvinylidene difluoride membrane for immunoblot analysis.

Kinase assays. In vitro phosphorylation assays were carried out in kinase buffer containing 25 mM Tris–HCl, pH 7.5, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 50 μM ATP, and 10 μCi [γ-^{32}P] ATP (5000 Ci/mM), at 30 °C for 30 min. The reactions were initiated by adding 500 ng AtCRK3, or AtCRK3P1, terminated by adding 1/5 volume 5× Laemmli sample buffer, and analyzed by 10% SDS–PAGE. After staining with 0.1% Coomassie Brilliant Blue, the gels were vacuum-dried and exposed to X-ray film at −80 °C. For time course assays, aliquots for the zero time point were taken immediately after the addition of AtCRK3 to initiate the reaction. Reactions were terminated by adding 1/5 volume 5× SDS–PAGE sample buffer and analyzed by SDS–PAGE. After staining with 0.1% Coomassie Brilliant Blue, the substrate bands were collected and the ^{32}P incorporation was determined by liquid scintillation counting (Beckman LS 6500). The experiments were repeated three times in duplicate.

Transient expression in onion epidermal cells. The full length open reading frame of *AtCRK3* and *AtGLN1;1* was cloned into pUC-GFP vector. Protocols for transient expression were according to the previous methods described [23].

In vivo co-immunoprecipitation from transformed Arabidopsis suspension cultured cells. The binary vectors were introduced into *Agrobacterium* strain GV3101 by electroporation and used in co-transformation experiments to express pairwise combinations of proteins in cultured *Arabidopsis* cells, as described previously [24]. *Arabidopsis* cells expressing the constructs were harvested at 6 days after *Agrobacterium*-mediated transformation. The frozen cells were homogenized to powder in the presence of liquid nitrogen and resuspended on ice in extraction buffer (1 ml/g) containing 50 mM Tris–HCl, pH 7.6, 10% glycerol, 0.5% NP-40, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 20 μl/ml Sigma plant protease inhibitor mix. After preparation of a cleared lysate by centrifugation (6000*g* for 15 min at 4 °C), the protein concentration in the supernatant was determined. Then the extract was supplemented with 150 mM NaCl and was subjected to immunoaffinity purification. The protein extract was pre-cleared with 50 μl of protein G-Sepharose (Sigma; equilibrated in IPW buffer: 50 mM Tris–HCl, pH 7.6, 150 mM NaCl, and 0.1% NP-40; Sigma) by continuous shaking at 4 °C for 2 h. The protein extract was subjected to immunoaffinity purification and immunodetection as described [24].

Locus numbers of the genes indicated in the article are as follows: At5g37600 (*AtGLN1;1*), At2g46700 (*AtCRK3*), and At2g41140 (*AtCRK1*).

Results

Identification of AtGLN1;1 as an AtCRK3 interacting protein by yeast two-hybrid screen

To identify proteins capable of interacting with AtCRK3, the N-terminal region of the enzyme was fused to the GAL4-DNA binding domain and used as a bait to screen an activation domain-tagged cDNA library prepared from *Arabidopsis*. Among total of 7–8× 10^6 co-transformants, AtGLN1;1 lacking the N-terminal 10 amino acids was identified under selective conditions and LacZ activity, suggesting AtCRKP1 could interact with almost full length AtGLN1;1 protein in yeast.

To further map the potential binding regions for AtGLN1;1 and AtCRK3 respectively, a series of constructs were employed in yeast two-hybrid system (Fig. 1A). As shown in Fig. 1B, none of the constructs used could auto-activate itself. Both full length and partial form (AtCRK3P1) of AtCRK3 could interact with AtGLN1;1 (full length) and AtGLNP2 (catalytic domain of AtGLN1;1) in yeast, but not with AtGLNP1 (the N-terminal beta-grasp domain of AtGLN1;1). Furthermore, AtCRKP3 (131–401, kinase domain of AtCRK3) alone was able to interact with AtGLNP2 (Fig. 1B), indicating that the kinase domain of AtCRK3 and the catalytic domain of AtGLN1;1 were respectively responsible for the interaction of these two proteins. When the ATP-binding site of the kinase domain was deleted, AtCRK3P4 (175–401) lost its ability to interact with AtGLN1;1 (1–356) and AtGLNP2 (103–356), suggesting that the kinase activity might be necessary for the interaction.

The overall amino acid sequence of the CRK proteins is highly conserved [2]. This raises the question whether particular CRK isoforms have distinct biological functions by selectively regulating their specific substrates. To address this question, AtCRK1 as a representative of other *Arabidopsis* CRKs was used to test whether it interacts with AtGLN1;1 or not. The results revealed that the newly identified interacting protein AtGLN1;1 only interact with AtCRK3, but failed to interacted with AtCRK1P1,

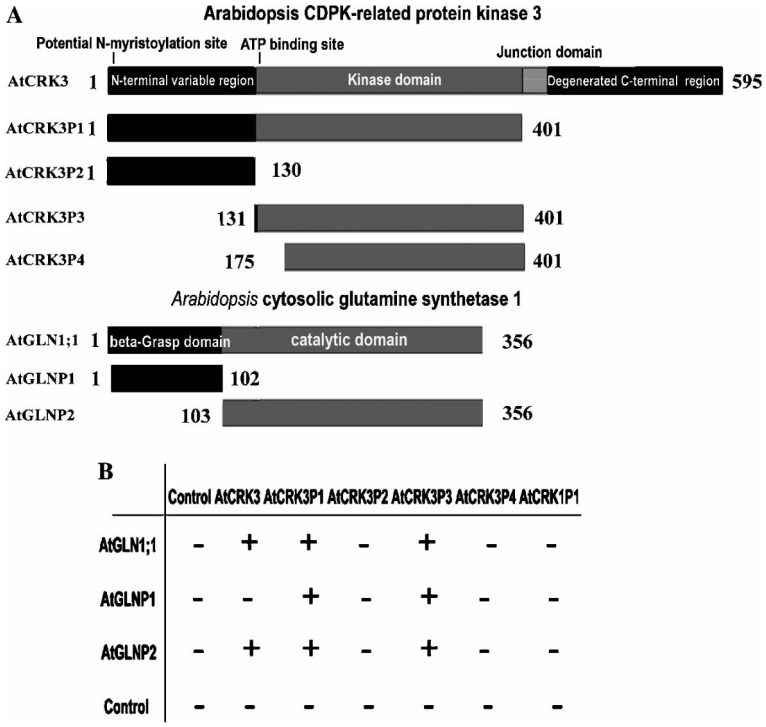

Fig. 1. Yeast two-hybrid assays for the interaction between AtCRK3 and AtGLN1;1. (A) Scheme of the full length and truncated forms of AtCRK3 and AtGLN1;1 used in the experiments. (B) Results of yeast two-hybrid assays for the interaction specificity and interaction domain between AtCRK3 and AtGLN1;1. Empty vector PGADT7 and PGBKT7 were used as control, respectively.

suggesting that the observed interaction is specific to certain CRK to some extent.

Direct interaction between AtCRK3 and AtGLN1;1 in vitro

To further confirm the interaction between AtCRK3 and AtGLN1;1, we carried out the in vitro protein binding assay with purified recombinant proteins of AtCRK3 and AtGLN1;1. The AtCRK3 and AtCRK3P1 were expressed as 6× His fusion protein with N-terminal c-myc epitope in sf9 insect cells. Both full length form and catalytic domain (AtGLNP2) of AtGLN1;1 were expressed as 6× His fusion proteins with C-terminal HA epitope in *E. coli* cells.

The interaction between AtCRK3 and AtGLN1;1 was then tested in vitro using an immunoprecipitation assay with the purified proteins. The crude protein extracts from uninfected insect cells and *E. coli* cells transformed with empty pET28a were used as control respectively. Because Mg^{2+} forms a complex with ATP and this complex appears to be one protein kinase substrate [6], Mg^{2+}/ATP was also used to test their effects on the interaction. When anti-HA antibody was used first to immunoprecipitate the HA-tagged AtGLN1;1 or AtGLNP2 proteins, the immunoblotting results indicated that both AtCRK3 and AtCRKP1 could be detected while the control has no positive signal, which confirmed that both AtCRK3 and AtCRKP1 could directly and specifically interact with AtGLN1;1 and AtGLNP2 in vitro (Fig. 2A). The results also revealed that addition of Mg^{2+}/ATP greatly enhanced such interactions (Fig. 2A), indicating that the interaction might be a phosphorylation enhanced process. When anti-myc was used first to immunoprecipitate the c-myc-tagged AtCRK3 or AtCRK3P1 protein, similar results were obtained (Fig. 2B).

In vivo interaction between AtGLN1;1 and AtCRK3

The AtCRK3–AtGLN1;1 interaction was further supported by the results of gene expression and protein localization in the levels of tissues and cells, respectively. First, the tissue expression patterns of *AtGLN1;1* and *AtCRK3* were examined for it is generally considered that their gene expression domains should overlap if two proteins interact in vivo. Our results showed that *AtCRK3* and *AtGLN1;1* show a similar expression pattern, although the mRNA level of *AtGLN1;1* was much higher than the level of *AtCRK3*. They both ubiquitously expressed in all tested organs including roots, flowers, leaves, stems, and siliques while have more abundant expressions in siliques and roots (Fig. 3A). In addition, subcellular localization analyses of AtCRK3 and AtGLN1;1 with GFP as a marker revealed both AtCRK3-GFP and AtGLN1;1-GFP fusion proteins could localize in the cytoplasm (Fig. 3B).These similar gene expression patterns and protein localizations provide the base for their possible interaction.

Further evidence for the AtCRK3–AtGLN1;1 interaction came from the observations that AtCRK3 could be co-immunoprecipitated with AtGLN1;1 in vivo.

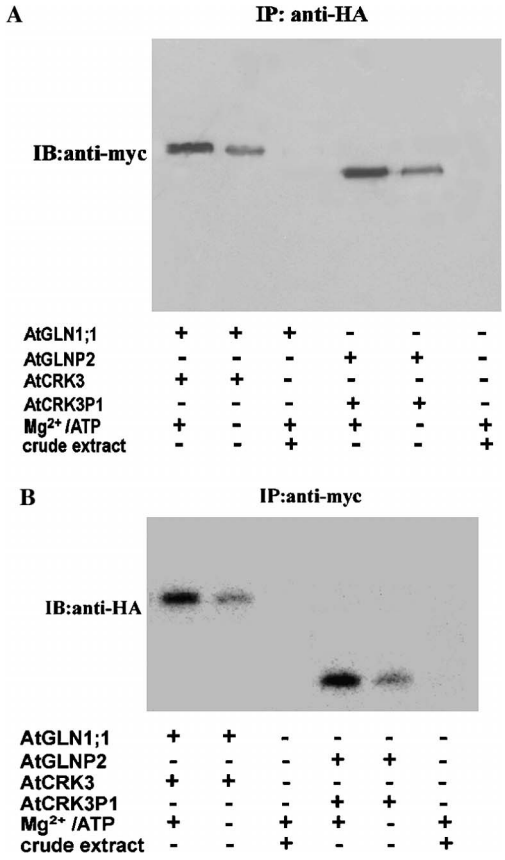

Fig. 2. In vitro binding assays for AtGLN1;1 and AtCRK3. (**A**) In vitro protein binding assays for binding of AtCRK3 or AtCRK3P1 to immunoprecipitated AtGLN1;1 or AtGLNP2. Mg^{2+}/ATP were added to test their role in the interactions. The crude protein extract from *E. coli* cells transformed with empty pET28a was used as control. (B) In vitro protein binding assays for binding of AtGLN1;1 or AtGLNP2 to immunoprecipitated AtCRK3 or AtCRK3P1. The crude protein extract from sf9 insect cells was used as control. The symbols + and − indicate the presence or absence of constructs in the transformation assays. IP and IB refer to the antibodies used for immunoprecipitation (IP) and immunoblot (IB), respectively.

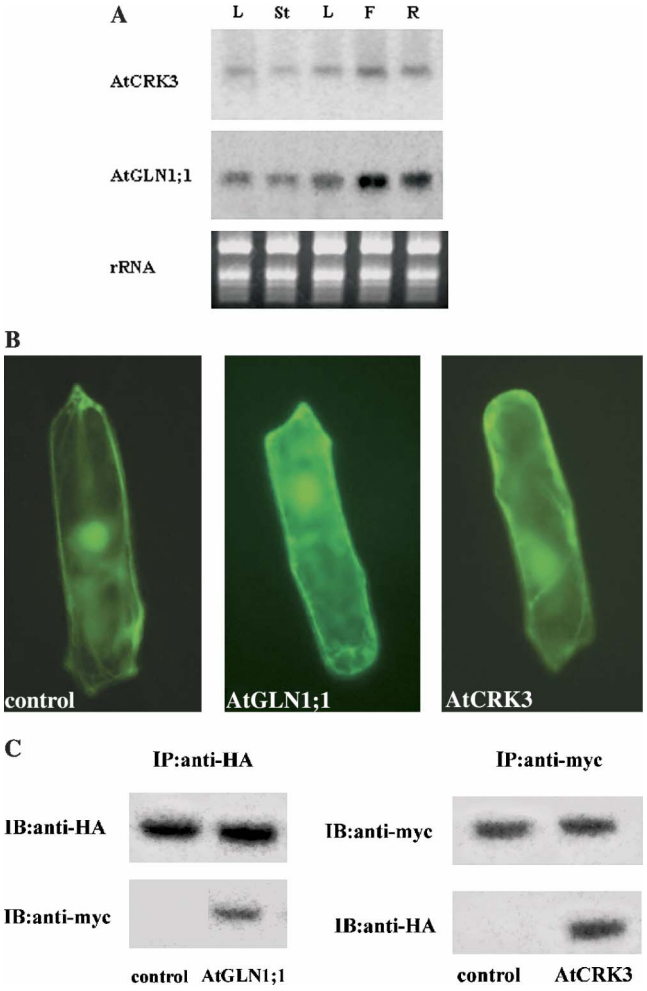

Fig. 3. AtGLN1;1 and AtCRK3 could interact in vivo. (A) Northern-blot analyses for *AtCRK3* and *AtGLN1;1* expression in different organs. Roots (R), stems (St), leaves (L), flowers (F), and siliques (S) were harvested from 4- to 8-week-old plants. (B) Subcellular localization of AtGLN1;1-GFP and AtCRK3-GFP fusion proteins in onion epidermal cells. Soluble localization of GFP protein alone was used as control. (C) Interaction of AtGLN1;1 and AtCRK3 in co-transformed *Arabidopsis* cells. *Arabidopsis* suspension cultured cells were co-transformed with AtGLN1;1-myc and HA-AtCRK3 expression vector. Co-transformation of AtGLN1;1-myc with empty pPCV812 vector and HA-AtCRK3 with empty pPCV002 vector were used as control respectively. From each double transformed cell line, 1 mg protein extract was subjected to immunoaffinity purification with either an anti-HA antibody (left) or anti-c-myc antibody (right) immobilized to protein G-Sepharose.

HA-tagged AtCRK3 and c-myc-tagged AtGLN1;1 binary expression vectors were first co-expressed in suspension cultured *Arabidopsis* cells, then co-immunoprecipitation assay was performed. Empty vector pPPCV012 or pPPCV812 was used as control, respectively. The whole cell lysates were immunoprecipitated with HA antibody followed by immunoblot analysis with c-myc antibody or immunoprecipitated with c-myc antibody followed by immunoblot analysis with HA antibody. As shown in Fig. 3C, AtCRK3 was specifically co-immunoprecipitated with AtGLN1;1 and vice versa. These results suggest that AtCRK3 and AtGLN1;1 associate in *Arabidopsis* cells.

Phosphorylation of AtGLN1;1 by AtCRK3

The fact that Mg^{2+}/ATP-enhanced interaction of AtCRK3 and AtGLN1;1 prompted us to investigate whether AtGLN1;1 was a substrate for the AtCRK3. Phosphorylation assays indicated that both AtCRK3 and AtCPK3P1 proteins were able to phosphorylate full length AtGLN1;1 protein and the truncated form AtGLNP2 (Fig. 4, lanes 1–4), which is consistent with the protein interaction results. Such phosphorylation might be specific to AtCRK3 because the AtCRK1 or AtCRKP1 proteins cannot phosphorylate either AtGLN1;1 or AtGLNP2 (Fig. 4, lanes 5 and 6). A series of biochemical determinations were also carried out for this phosphorylation. The results showed that phosphate incorporation into AtGLN1;1 was linear up to 30 min. The K_m values for AtGLN1;1 are 20.4 ± 1.1 μM, which conforms to criteria

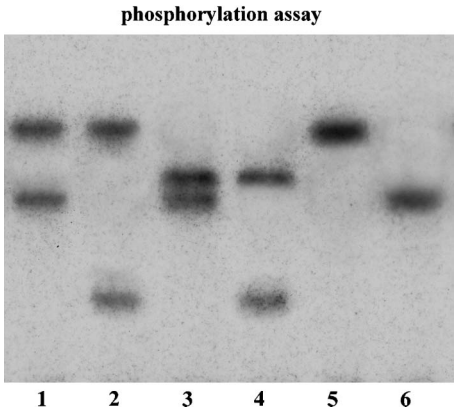

Fig. 4. Phosphorylation of AtGLN1;1 by AtCRK3. Purified AtGLN1;1, AtGLNP2, AtCRK3, and AtCRK3P1 proteins were used for the phosphorylation assays. AtCRK1P1 and AtCRK1P1 were used for kinase specificity tests. lane 1, AtCRK3 + AtGLN1;1; lane 2, AtCRK3 + AtGLNP2; lane 3, AtCRK3P1 + AtGLN1;1; lane 4, AtCRK3P1 + AtGLNP2; lane 5, AtCRK1 + AtGLN1;1; lane 6, AtCRK1P1 + AtGLN1;1.

for consideration of a protein as a potential CDPK substrate ($K_m \leqslant 50$ μM) [4].

Expression of AtCRK3 and AtGLN1;1 during leaf senescence

The results above have demonstrated that AtCRK3 could interact and phosphorylate AtGLN1;1. Additional work is needed to determine a biologically relevant effect of such phosphorylation. Changes in gene expression frequently reflect the biological function of the genes in different processes. The role of GS1 in *Arabidopsis* leaf senescence has been discussed [25], thus we examined here the expression patterns of the *AtCRK3* and *AtGLN1;1* gene under natural leaf development and senescing conditions. The results (Fig. 5A) showed that the *AtCRK3* and *AtGLN1;1* genes are both induced in leaves at early senescent stage (S1), and the mRNA level is maintained at a comparable level (S2 and S3). In addition, *AtGLN1;1*, was expressed higher in the adult leaf and cauline leaf than in the juvenile leaf. It may be indicated that the expression of *AtGLN1;1* was gradually induced with leaf development other than only induced by senescence. It is interesting that the expressions of *AtCRK3* and *AtGLN1;1* in senescent stems are also higher than in young stems, suggesting their possible roles in nutrient transport during senescence.

In order to investigate whether these two genes are also associated with artificially induced leaf senescence, dark-induced senescence of detached leaves was used as an in vitro experimental system to study the expression of the genes. As shown in Fig. 5B, both *AtCRK3* and *AtGLN1;1* were induced after 2 days dark treatment, and maintained at a comparable level up to 6 days, similar to the results from natural senescence. These results suggest that the two genes might be physiologically relevant in leaf senescence.

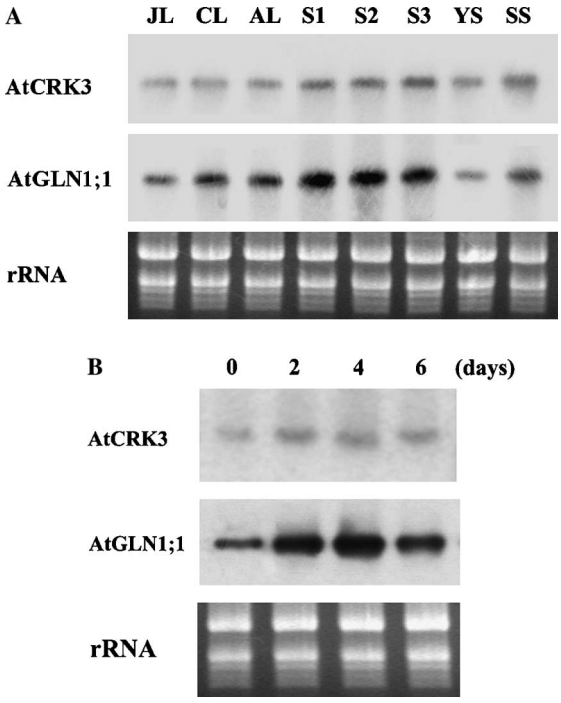

Fig. 5. Expression of *AtCRK3* and *AtGLN1;1* during senescence. (A) Northern-blot analyses of *AtCRK3* and *AtGLN1;1* expression during leaf and stem growth and senescence. Lanes contain 20 μg of total RNA extracted from juvenile leaves (JL), cauline leaves (CL), adult leaves (AL), senescent leaves (S1, S2, and S3), young stems (YS), and senescent stems (SS) of *Arabidopsis*. (B) Expression of *AtCRK3* and *AtGLN1;1* during dark induced senescence. The detached leaves of *Arabidopsis* were incubated in permanent darkness on wet filter paper for up to 7 days. Samples were collected from 2, 4, and 6 days.

Discussion

Despite the rapid progress in identifying CDPK components, physiological functions for CRKs still remain largely unknown in recent years [4]. In this study, we provide evidences that AtGLN1;1 is a potential CRK substrate, which may be involved in the regulation of leaf senescence or nitrogen remobilization. This is the first report on the substrate of plant CRKs.

Up to now, all kinds of traditional biochemical and genetic approaches have been used to isolate kinase substrates. More recently, proteomics, functional genomics, bioinformatics or chemical genomics will revolutionize the way that scientists approach the comprehensive characterization of the protein kinases in plants [26,27]. Yeast two-hybrid system was first used to isolate substrate of plant CDPK by Patharkar and Cushman [28], which was also demonstrated to be successful in the identification of the binding protein of NtCDPK1 [29]. In this report, we also identified AtGLN1;1 as a potential substrate of AtCRK3. So it seems that yeast two-hybrid system is a useful method in plant protein kinase research.

Although CDPKs have been shown to phosphorylate a large number of protein substrates in vitro, limited information is available regarding substrate specificity in

vivo [2]. Sheen showed that only two out of eight CDPK isoforms could activate expression from a stress- and ABA-regulated promoter (HVA-1) in a transient expression system, indicating the different CDPK isoforms have different substrate specificities and distinct functions [30]. Our results reveal that AtGLN1;1 can interact specifically with and be phosphorylated by AtCRK3, but does not interact with AtCRK1, indicating a specific interaction between AtGLN1;1 and AtCRK3. These results suggest that CRK isoforms may have functional specificity. In addition, the AtCRK3 interaction domain was localized in the kinase domain, demonstrating that the specificity may be mainly due to this domain, in spite of the fact that the amino acid residues exposed in the kinase domain were highly conserved. The result is consistent with the reports that the PK domain of AtCDPK1 is required and sufficient to recognize specific protein substrates mediating stress signal transduction [30]. The finding that a defect in binding of ATP could lead to defective binding of substrate from our yeast two-hybrid data is similar to the results from animal CaMKII [31]. Since an ordered substrate-binding mechanism has been proposed in the case of CaMKII in which ATP binds the enzyme first and then the substrate. The enhanced binding of these two proteins by additional Mg^{2+}/ATP in our results also support the role of Mg^{2+}/ATP for the interaction of AtCRK3 and AtGLN1;1.

As a key enzyme in the nitrogen assimilatory process, the complete understanding of the mechanisms controlling GS activity in plants is of crucial importance. It has been generally considered that transcriptional regulation of the GS gene family is the main regulatory point controlling GS activity in plant cells [15]. However, the accumulating evidences have indicated the existence and importance of post-translational mechanisms like reversible protein phosphorylation regulating GS activity. Phosphorylation of plant GS was first observed by Moorhead et al. [18]. Recently, this mechanism has been shown to be involved in the regulation of cytosolic GS in senescing leaves of *Brassica napus* [17] and green alga *Chlamydomonas reihardtii* [19], as well as plastidial GS from *Nicotiana tabacum* [20] and *Hordeum vulgare* [16], but the requisite protein kinase has not been identified. We first identified a protein kinase, AtCRK3 which could interact and phosphorylate GS1.

Leaf senescence is a complex process involving the coordinated action of many degradative pathways that lead to maximum recovery of leaf components to support further development [32,33]. Nitrogen remobilization from the old leaves is one of the most important functions [15]. During senescence, the nitrogen is primarily released from protein breakdown and nucleic acid metabolism [34], which was estimated to be the most recycled nutrient (>90% in *Arabidopsis* of total nitrogen) during senescence [34]. Nitrogen is converted to transportable amino acids, usually in the form of glutamine [17], which can be transported via phloem from senescencing leaves to sinks within the plant [15]. For GS1 in leaves and stem is localized primarily in the phloem elements, it is postulated that GS1 may function to generate glutamine to participate in vascular nitrogen transport during leaf senescence [35]. The senescence-related increase of GS1 gene expression has been observed previously in several different plants [35–37]. A positive correlation between the increase in leaf proteolytic activity and the expression of two senescence marker genes (glutamine synthetase and glutamate dehydrogenase) has also been suggested by other studies [38]. The direct evidence for the role of GS1 in regulating senescence comes from the result that senescence occurred earlier in plants over-expressing an exogenous GS1 gene [39]. In addition, calcium signaling may be also an important component in the regulation of senescence processes. The involvement of calcium in many types of cell death has been described, and increased levels of calcium ions have been observed to correlate with senescence of parsley mesophyll cells [40]. Our results suggest that expressions of *AtCRK3* and *AtGLN1;1* were induced by leaf senescence, suggesting their possible roles in the regulation of nitrogen remobilization during senescence.

In summary, AtGLN1;1 has been identified as an interaction protein of AtCRK3, and it is proved to be phosphorylated by this kinase in our work. We also found both of them are induced in leaves at early senescent stage. Future work is needed to understand the phosphorylation regulation of AtGLN1;1 in detail in vivo. Biological functional analysis for AtCRK3 and AtGLN1;1 will be also needed to define the possible effects of this interaction in nitrogen remobilization.

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