Biochemical and Structural Properties of Cyanases from Arabidopsis thaliana and Oryza sativa

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Abstract

Cyanate is toxic to all organisms. Cyanase converts cyanate to CO₂ and NH₃ in a bicarbonate-dependent reaction. The biophysical functions and biochemical characteristics of plant cyanases are poorly studied, although it has been investigated in a variety of proteobacteria, cyanobacteria and fungi. In this study, we characterised plant cyanases from Arabidopsis thaliana and Oryza sativa (AtCYN and OsCYN). Prokaryotic-expressed AtCYN and OsCYN both showed cyanase activity in vitro. Temperature had a similar influence on the activity of both cyanases, but pH had a differential impact on AtCYN and OsCYN activity. Homology modelling provided models of monomers of AtCYN and OsCYN, and a coimmunoprecipitation assay and gel filtration indicated that AtCYN and OsCYN formed homodecamers. The analysis of single-residue mutants of AtCYN indicated that the conserved catalytic residues also contributed to the stability of the homodecamer. KCNO treatment inhibited Arabidopsis germination and early seedling growth. Plants containing AtCYN or OsCYN exhibited resistance to KCNO stress, which demonstrated that one role of cyanases in plants is detoxification. Transcription level of AtCYN was higher in the flower than in other organs of Arabidopsis. AtCYN transcription was not significantly affected by KCNO treatment in Arabidopsis, but was induced by salt stress. This research broadens our knowledge on plant detoxification of cyanate via cyanase.

Introduction

Cyanate is generated by the chemical industry and mining wastewater [1]. It is well established that urea is in equilibrium with ammonium cyanate in solution and can spontaneously transform to cyanate [2], and photo-oxidation of cyanide in solution also produces cyanate [3,4]. Cyanate is toxic. Isocyanate, the active form of cyanate, reacts with amino and carboxyl groups and carbamoylates amino acids, proteins and other molecules, thereby altering their structure, charge and function [5,6].

Cyanase (EC 4.2.1.104, also known as cyanate lyase or cyanate hydrolase) catalyses a bicarbonate-dependent reaction decomposing cyanate to ammonia and bicarbonate [7]. Cyanase was first identified in Escherichia coli and has been characterised in detail in this bacterium [7,8,9,10,11,12,13]. Subsequently, the enzyme was discovered and characterised in proteobacteria [14,15,16,17,18], cyanobacteria [19,20], fungi [21] and plants [22]. The monomer of E. coli cyanase is a 17 kDa subunit [11] and the functionally active enzyme is a homodecamer of five dimers [8]. Three catalytic residues (Arg96, Gln99 and Ser122) are conserved in cyanase sequences [14,21], and the enzyme is competitively inhibited by a number of monovalent anions [10]. Cyanase activity is affected by pH and temperature [12,21]. Among living organisms, cyanase plays a role in detoxification of cyanate and cyanide [23]. Because the direct products of cyanase action are ammonia and bicarbonate, cyanate is utilised as a nitrogen and carbon source in some organisms [17,24].

Plant cyanase may play roles in different physiological pathways. In Medicago truncatula, the transcription level of the putative cyanase gene is lower in nodules than in roots [25]. In Suaeda aegyptica, the putative cyanase is upregulated in the leaf during salt accumulation [26]. However, only the cyanase gene in Arabidopsis has been cloned [22]. Because few studies on plant cyanases have been published, more information is needed to understand the roles of cyanase in plants. This study is focused on cyanases in the model plants Arabidopsis thaliana and Oryza sativa.

The objective was to understand the biochemical and structural properties and physiological roles of cyanases from the two species.

Results

Isolation of cDNAs encoding cyanases from Arabidopsis thaliana and Oryza sativa

To study cyanases in plants, we performed a BLAST-P search for proteins homologous to E. coli cyanase in the public NCBI Entrez databases, and located 12 putative plant cyanases in addition to that of A. thaliana (Table 1). The multiple amino acid sequence alignments showed that all of the cyanases were highly conserved in the C-terminal region (Figure 1). The plant cyanases shared high sequence identity (35.8%) and similarity (87.4%). The sequences of the six Dicotyledoneae cyanases exhibited 68.4%
identity and 94.7% similarity, while the sequences of the three Monocotyledoneae cyanases showed 91.2% identity and 100% similarity. AtCYN and OsCYN shared 70.5% sequence identity and 80.0% similarity. In particular, the three catalytic residues (Arg96, Glu99 and Ser122) in Escherichia coli cyanase (EcCYN) were conserved in all putative cyanases from fungi, animals and plants.

An unrooted phylogenetic tree representing relationships among these cyanases is presented in Figure 2. The two main clusters represent Dicotyledoneae and Monocotyledoneae cyanases. It indicated that, although the plant cyanase sequences were highly conserved, there has been genetic divergence between dicot and monocot cyanases. Therefore, we cloned the cDNAs of AtCYN and OsCYN, which encode two 168 aa proteins.

### Effects of pH and temperature on the enzyme activity of heterologously expressed AtCYN and OsCYN

We produced His-tagged recombinant CYN proteins in *E. coli*. Fortunately, the proteins are soluble and could be purified. The enzyme activities of OsCYN and AtCYN were measured. In the standard assay solution at 27°C, the activity of OsCYN was

| Species Abbreviation | Accession No. | Length | Taxonomic classification |
|----------------------|--------------|--------|-------------------------|
| Arabidopsis thaliana  | A tha        | NP_188991 | 168 aa | Planta; Dicotyledoneae |
| Glycine max          | G max        | ACU13914 | 165 aa | Planta; Dicotyledoneae |
| Ricinus communis     | R com        | EEF29508 | 164 aa | Planta; Dicotyledoneae |
| Vitis vinifera       | V Vin        | XP_002285393 | 164 aa | Planta; Dicotyledoneae |
| Medicago truncatula  | M tru        | ACJ8540 | 165 aa | Planta; Dicotyledoneae |
| Populus trichocarpa  | P tri        | XP_002315578 | 162 aa | Planta; Dicotyledoneae |
| Oryza sativa         | O sat        | NP_001064827 | 168 aa | Planta; Monocotyledoneae |
| Zea mays             | Z may        | NP_001150815 | 166 aa | Planta; Monocotyledoneae |
| Sorghum bicolor      | S bic        | XP_002467098 | 166 aa | Planta; Monocotyledoneae |
| Picea sitchensis     | P sit        | ABK22377 | 161 aa | Planta; Gymnospermae |
| Physcomitrella patens| P pat        | XP_001780115 | 154 aa | Planta; Bryophyta |
| Phaeodactylum tricornutum CCAP 1055/1 | P pat CCAP | XP_002177029 | 193 aa | Planta; Bacillariophyta |
| Thalassiosira pseudonana CCMP1335 | T pse CCMP | XP_002295609 | 205 aa | Planta; Bacillariophyta |
| Trichinella pseudosporalis | T pse | ABR10530 | 181 aa | Nematoda |
| Sordaria macrospora  | S mac        | CAO79555 | 164 aa | Fungi |
| Synchocystis sp.PC6803 | Syn sp.       | NP_442379 | 149 aa | Cyanobacteria |
| Escherichia coli str. K-12 substr. MG1655 | E col | NP_414874 | 156 aa | Proteobacteria |

![Figure 1. Alignment of catalytic regions of cyanases from fungus, plant and bacterial species.](image)

**Table 1.** List of cyanases from plants, fungi and bacteria.

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The biochemical properties of the enzymes were shown in Table 2. Activities of the cyanases of *E. coli* and *Sordaria macrospora* are affected by pH and temperature [12,21]. Therefore, activities of AtCYN and OsCYN were measured across a pH range between 4.8–8.8. As shown in Figure 3A, the optimum pH for AtCYN activity was 7.7 (2.286 U mg⁻¹). At pH 6.7 and 8.8, AtCYN retained >75% activity, but at a low pH (pH 5.7 and 4.8) AtCYN showed no activity. These experiments demonstrated that AtCYN activity is greatly affected by pH. However, OsCYN activity was only slightly affected by pH. The OsCYN activity ranged from 0.303 U mg⁻¹ at pH 4.8 and 0.633 U mg⁻¹ at pH 7.7.

To examine the influence of environmental temperature on cyanase activity, we measured enzyme activity at 19°C, 27°C and 34°C. Figure 3B showed that the activities of both enzymes increased concomitantly with increasing temperature. The activities of both AtCYN and OsCYN were >2-fold higher at 34°C than that at 19°C. Thus, the effect of temperature on the activities of AtCYN and OsCYN was similar, whereas the effect of pH differed.

### Involvement of ATCYN and OsCYN in cyanate decomposition in vivo

To study the function of cyanases in plants, we obtained four T-DNA insertion mutants of *Arabidopsis thaliana* from the Arabidopsis Biological Resource Center (Figure 4A), but we were unable to obtain *cyn* mutants of *Oryza sativa* for study. AtCYN transcripts were detected in the mutant plants (Figure 4B). In the *cyn-1* line, the transcript level was reduced to 30–40% of that in Col 0. AtCYN transcription was not detected in the *cyn-7* line. We also generated transgenic lines CaMV35S:HA:AtCYN/*cyn-7* (1#, 2# and 3#) and CaMV35S:HA:OsCYN/*cyn-7* (4#, 5# and 6#) in the *cyn-7* mutant background with CYN cDNAs from Arabidopsis and rice. AtCYN transcripts in three independent transgenic lines CaMV35S:HA:AtCYN/*cyn7* lines (1#, 2# and 3#) were 15–65% the level of Col 0 (Figure 4C). As shown in Figure 4D, HA:AtCYN and HA:OsCYN proteins were detected in these transgenic lines.

We treated wild-type and mutant plants with four concentrations of potassium cyanate (KCNO; Figure 5). The wild-type seeds germinated and grew normally at 0.5 mM and 1 mM KCNO but showed a slow growth at 2 mM KCNO. The knock-out mutant *cyn-7* seeds did not germinate at 1 mM and 2 mM KCNO, and grew very slowly at 0.5 mM KCNO. The knock-down mutant *cyn-1* seeds germinated at concentrations less than 1 mM KCNO, but seedlings grew more slowly than Col 0 seedlings. This experiment demonstrated that cyanate treatment inhibited germination and early seedling growth of *Arabidopsis*, and that wild-type plants showed resistance to cyanate stress. In *AtCYN* knock-down and knock-out mutant plants, the resistance to cyanate stress was weaker or lost completely.

We also treated transgenic plants with KCNO. The molecular complementation experiment was undertaken to determine whether the *cyn-7* mutant could be rescued by constitutive
expression of HA:AtCYN and HA:OsCYN. As Figure 5 shows, the resistance to cyanate stress of the transgenic lines was intermediate to that of the wild-type and the knock-out mutant cyn-7. Lines 2# and 5#, for which the cyanase expression levels were higher than those of other lines (Figure 4D), showed higher resistance to cyanate. In particular, line 2# showed almost identical resistance as the cyn-1 mutant, which corresponded with similar AtCYN transcript levels between the two lines (Figure 4B, C). The complementation experiment confirmed that cyanase contributes to Arabidopsis resistance to cyanate stress and provided direct evidence that plant cyanases decompose cyanate in vivo.

Structural properties of AtCYN and OsCYN
The active E. coli cyanase is a homodecamer of 5 dimers comprising 17 kDa subunits, and has 3 catalytic residues Arg96, Glu99 and Ser122 [8]. To study the structural properties of AtCYN and OsCYN, we performed homology modelling using the SWISS-MODEL service. As expected, the program selected the crystal structure of E. coli cyanase monomer (chain J, PDB code 1dw9J) as a template. As Figure 6A shows, the backbones of the predicted structures of AtCYN and OsCYN were both similar to the crystal structure of the EcCYN monomer. Although the predicted secondary structure near the Ser at the C-terminus of ACYN was different from that of EcCYN, the positions of the three conserved catalytic residues Arg, Glu and Ser seem unchanged (Figure 6B, C). This suggests that AtCYN and OsCYN have similar monomer structures. Therefore, similarity of the quaternary structures and catalytic sites was expected.

Table 2. Biochemical characteristics of heterologously expressed AtCYN and OsCYN.

| Kinetic Parameter | AtCYN | OsCYN |
|-------------------|-------|-------|
| Km NaHCO₃ (mM)    | 0.79  | 0.63  |
| Km KTN (mM)      | 0.94  | 7.38  |
| Vmax (nmol·mg⁻¹·min⁻¹) | 3820 | 982   |
| Vmax (nmol·mg⁻¹·min⁻¹) | 3980 | 2080  |
| Optimum pH       | 7.7   | 5.7   |

The apparent Km and Vmax values were calculated by double-reciprocal plots. 
*The cyanase reaction was assayed at 27°C, pH 7.7, in the presence of 5 mM KCN. 
**The cyanase reaction was assayed at 27°C, pH 7.7, in the presence of 5 mM NaHCO₃.

Figure 4. Identification of Atcyn mutant plants and transgenic plants. (A) Schematic diagram of the AtCYN gene and the T-DNA insertion positions. Gray boxes represent exons. Lines above the gene indicate T-DNA insertion positions. Accession numbers of the Atcyn mutant lines was listed in Table 4. (B) Northern blot analysis of AtCYN transcripts in cyan mutants. Total RNA was isolated from 14-day-old seedlings. Different homozygous individuals identified were analyzed in different lanes. Blot signals (indicated by the arrow) were quantified with ImageJ version 1.4 software and the values are presented below each lane. The 5S rRNA was visualized with ultraviolet light and was the loading control. (C) Quantitative RT-PCR analysis of AtCYN transcripts in Col 0, cyn7 and transgenic plants. Error bars represent the standard deviation of three biological replicates. (D) Semi-quantitative analysis of HA:AtCYN and HA:OsCYN in transgenic plants using western blotting (WB). Blot signals (indicated by the arrow) were quantified with ImageJ and the values are presented below the lanes. The large subunit of Rubisco was visualised by Coomassie Brilliant Blue (CBB) and was the total protein loading control (indicated by the arrow).

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We performed coimmunoprecipitation experiments to identify the self-interaction of AtCYN and OsCYN in vivo. Using an Agrobacterium infiltration assay, we transiently expressed HA-tagged and Flag-tagged CYN (AtCYN and OsCYN) in N. benthamiana. As shown in the upper image in Figure 7A, Flag:AtCYN coimmunoprecipitated with HA:AtCYN, and in the reciprocal experiment shown in the lower image HA:AtCYN coimmunoprecipitated with Flag:AtCYN. An identical pattern was shown by OsCYN (Figure 7B). Thus, the self-interaction of AtCYN and OsCYN was demonstrated. This indicates that either AtCYN or OsCYN could form a dimer or polymer in vivo.

Using the gel filtration assay, we calculated the molecular weights of His-tagged recombinant CYN proteins. His:AtCYN was 210.74 kDa with a 21.9 kDa monomer, and His:OsCYN was 211.72 kDa with a 22.0 kDa monomer (Figure 8A). This demonstrates that AtCYN and OsCYN are homodecamers in vitro.

We constructed two His-tagged AtCYN mutants each with a single residue change: AtCYN-E94L, in which Glu94 was replaced with Leu to break the proposed salt bridge, and AtCYN-S117A, in which Ser117 was replaced with Ala. The calculated molecular weights of monomers for both His-tagged mutant proteins were 21.9 kDa. However, gel filtration showed the molecular

Figure 5. Decomposition of cyanate by AtCYN and OsCYN in vivo. Seeds were plated on MS medium containing either 0 mM, 0.5 mM, 1 mM or 2 mM KCNO. Plates were incubated at 4°C for 3 days and then transferred to a growth chamber for 7 days. doi:10.1371/journal.pone.0018300.g005

Figure 6. Homology modelling of AtCYN and OsCYN. (A) The predicted structures of AtCYN (blue) and OsCYN (magentas) were similar to the crystal structure of the EcCYN monomer (green). Ball-and-stick figures represent the conserved catalytic residues Arg96, Glu99 (B) and Ser122 of the EcCYN (C). Red dots indicate chloride ions. doi:10.1371/journal.pone.0018300.g006
The 7-day-old seedlings grew in the MS medium containing KCNO or NaCl were harvested. The \textit{AtCYN} transcript level in the seedlings treated with three concentrations of KCNO showed no significant difference compared to that in untreated plants (Figure 9B). However, the \textit{AtCYN} transcripts were induced by salt treatment (Figure 9C). We transferred the 7-day-old seedlings from the standard MS medium to the MS medium containing KCNO or NaCl, and harvested the samples at different time points. It was unexpected that the \textit{AtCYN} transcripts decreased during the first 12 h under both treatments and increased thereafter (Figure 9D). The \textit{AtCYN} transcripts were down regulated in the early stage of the treatment. But the continuous salinity stress induced \textit{AtCYN} transcripts, while the continuous KCNO stress did not. These data suggested that a complex regulatory mechanism could control the expression of \textit{AtCYN}.

### Discussion

To broaden our understanding of cyanases in general, we characterised cyanases from the model plants \textit{Arabidopsis thaliana} and \textit{Oryza sativa}. The activities of heterologously expressed \textit{AtCYN} and \textit{OsCYN} were measured in vitro, and \( K_m \) values for both enzymes were calculated (Table 2). However, the activities of endogenously expressed plant cyanases were not determined directly. We isolated \textit{cyn} mutant \textit{Arabidopsis} and transformed 35S:HA:AtCYN and 35S:HA:OsCYN constructs into \textit{cyn-7} mutant plants. In the knock-out mutant plants, seed germination and early seedling growth were inhibited by KCNO presenting in the culture medium. However, all plants containing \textit{AtCYN} or \textit{OsCYN} showed cyanate resistance, and there was a positive correlation between resistance and cyanase expression levels. This indicates that plant cyanases contribute to cyanate tolerance and are involved in decomposition of cyanate in the plant.

BLAST-P searches of the NCBI databases indicated that putative cyanases are produced in some plants (Table 1). Amino acid sequences of these plant cyanases were highly conserved as are the known cyanases in prokaryotes and fungi (Figure 1). Reconstruction of phylogenetic relationships among the known cyanases (Figure 2) provided evidence for a common evolutionary origin for plant cyanases. It is suggested that the conserved cyanases are derived from an ancient gene, which makes it possible to study inter- and intra-specific relationships using cyanase as a phylogenetic marker [28]. The high resolution crystal structure of \textit{E. coli} cyanase explains the structural and kinetic properties of the enzyme: the active enzyme is a homodecamer composed of five inactive dimmers, and catalytic residues were identified [8]. In our study, homology modelling showed the monomer structures of \textit{AtCYN} and \textit{OsCYN} were similar to that of EcCYN (Figure 6). And the similar active homodecamer of plant cyanases was confirmed (Figure 7 and 8). Analysis of \textit{AtCYN} mutants E94L and S117A confirmed the conserved catalytic residues, and indicated that not only the glutamate but also the serine contributes to the formation of the active homodecamer (Figure 8), which was not mentioned in the previous studies of EcCYN. And the difference may be explained by the different structure near the serine (Figure 6C).

The cyanases shared higher sequence identity and some similar properties, but we also found different properties between the both plant cyanases. Although the assay conditions differ in pH and concentration of substrate, \( K_m \) values of both plant cyanases (0.79 mM for \textit{AtCYN} and 0.63 mM for \textit{OsCYN}) are approximate to that of the characterized PpCYN (CynS of \textit{Pseudomonas pseudoalcaligenes}, 0.67 mM) and SmCYN (CYN1 of \textit{Sordaria macrospore}, 0.83 mM) [14,21]. However, the characterized cyanases...
Characterization of Plant Cyanases

Figure 8. Gel filtration and cyanase activities of His-tagged AtCYN, OsCYN and AtCYN mutants (E94L and S117A). (A) Gel filtration. High Molecular Weight (HMW) Standard: Thyroglobulin, 669 kDa; Ferritin, 440 kDa; Aldolase, 158 kDa; Conalbumin, 75 kDa and Ovalbumin, 43 kDa.

The biophysical roles of cyanases have been investigated in certain bacteria, but remain unclear. With regard to more complicated organisms such as plants, the present study contributes to the characterisation of two plant cyanases, but further studies are needed to understand the regulation and the exact roles of cyanase in plants.
Characterization of Plant Cyanases

Sequence analysis and homology modelling

Protein sequence data for cyanase genes from other organisms were obtained by BLAST-P searches of the National Center for Biotechnology Information (NCBI) Entrez databases [49]. Multiple amino acid sequence alignments were performed using Clustal X software [50]. An unrooted phylogenetic tree was constructed using the neighbour-joining method with Clustal X and visualised using TreeView [51].

The homology structure modelling of AtCYN and OsCYN was performed using Swiss Model Service [52] based on the structure of E. coli cyanase (PDB code 1dw9j) as a template. The resulting models were analysed using PyMOL program (Version 1.1).

cDNA cloning and vector construction

Total RNA was isolated from various tissues of A. thaliana and O. sativa using TRizol® Reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was performed using Superscript II reverse transcriptase (Invitrogen) and the primers AtCYN 3’ and OsCYN 3’ (Table 3). The primer pairs AtCYN 5’/3’ and OsCYN 5’/3’ were used for reverse transcription PCR. AtCYN and OsCYN cDNAs were cloned into pBluescript II SK(+) at the EcoR V site to obtain the entry vectors pBS-AtCYN and pBS-OsCYN. The BamHI and Xho I sites were used to clone AtCYN and OsCYN from the entry vectors into the pET15b expression vector (Novagen), resulting in pET-AtCYN and pET-OsCYN. The mutants E94L and S117A of pET-AtCYN were constructed with the KOD-Plus Mutagenesis Kit (Toyobo), using the mutagenesis primer pairs E94L FP/RP and S117A FP/RP (Table 3). The pET-ATCYN constructs were introduced into E. coli BL21 (DE3) cells (Stratagene). The Sph I and Xba I sites were obtained by ligating the cDNAs from the entry vectors into the pRLL2HA and pRLL2Flag vectors. The HAtCYN and Flag:OsCYN fragments were digested with Sph I and blunt-ended with T4 DNA polymerase, then inserted into the binary vector pWM101 at the Sma I site. Expression of HAtCYN and Flag:OsCYN was under control of the CaMV 35S promoter.

Table 3. List of primers used in this study.

| Primer name | Primer sequence |
|-------------|-----------------|
| AtCYN 5'    | 5'-ATGGAAGCGCGGAAAGAACAGAAGGT-3' |
| AtCYN 3'    | 5'-TCATTCGCTCCATGATCCTCCTTGGAGG-3' |
| OsCYN 5'    | 5'-ATGGAGGGCGCGCCGGGGGAGAGGG-3' |
| OsCYN 3'    | 5'-TCACATCGCTCGGAGCTGAGC-3' |
| E94L FP     | 5'-CTAGCAATGATGCATTTGTTGAGAGAAG-3' |
| E94L RP     | 5'-ACCAAAATGCATCACTGCTTC-3' |
| S117A FP    | 5'-GCGGCGGATATTTTGTATG-3' |
| S117A RP    | 5'-CATGATGCTCCTCACCAAACTTCC-3' |
| Lb1         | 5'-CGGATGATGTTTCTGCTGAAAAC-3' |
| Lp1         | 5'-TCCTTAACTGGCTGAGCTGGA-3' |
| Lp2         | 5'-TCCTCTTGTTGCTAGCTGAGCTGAC-3' |
| R1P         | 5'-AGGCCCCGCTTACTATATAC-3' |
| R1P2        | 5'-ACGAGAAAATGATCTGCAGAC-3' |
| R3P         | 5'-TCCTTAATGGATCAGTTCGAC-3' |
| AtCYN P5    | 5'-GCGGACAGACGCTAACCACAC-3' |
| AtCYN P6    | 5'-ATCCTTCATACGCTTGACAAA-3' |
| AIEF4AF     | 5'-GCGCATCCCTCAGGCTGTTGTC-3' |
| AIEF4AR     | 5'-GGGCAAAAGAGCTGGAAAAATGCT-3' |

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana lines of the Col 0 background and O. sativa L. cv. Zhonghua 11 were used. The T-DNA insertion mutant lines were obtained from the Arabidopsis Biological Resources Center, Ohio State University. Seeds were plated on Murashige and Skoog (MS) medium supplemented with 0.8% agar and 1% sucrose, incubated at 4°C for 3 days then transferred to a growth chamber with a 14 h light/22°C and 10 h dark/18°C regime. Nicotiana benthamiana plants were grown in a growth chamber under a 12 h light/25°C and 12 h dark/18°C regime.
pWM-CYN constructs were introduced into Agrobacterium tumefaciens strain GV3101 by electroporation.

Protein preparation and gel filtration assay

Escherichia coli BL21 (DE3) cells containing the pET-CYN constructs were cultured in LB medium and His-tagged recombinant proteins were induced by adding 0.5 mM isopropyl β-D-thiogalactoside for 1–3 hours at 37°C. Cells were harvested and sonicated at 4°C. First, affinity chromatography was performed to purify the His-tagged proteins using Ni²⁺ chelating columns, and then the samples were loaded onto a Superdex 200 10/300 GL column for gel filtration chromatography.

Cyanase activity assay

Cyanase activity in vitro was determined by monitoring ammonia formation in the reaction medium as described previously [12]. The standard assay solution was comprised of 50 mM potassium phosphate buffer (pH 7.7) containing 3 mM sodium bicarbonate and 2 mM potassium cyanate. The reaction was started by the addition of the cyanase and terminated by the addition of equal volumes of Nessler reagent after 1–10 min at 27°C. The amount of ammonia formed was determined by monitoring the absorbance at 420 nm within 10 min of adding Nessler reagent. One unit of cyanase was defined as the amount of enzyme that catalyses the formation of 1 μmol NH₃ min⁻¹ under the assay conditions. To allow for the influence of temperature, the reaction was assayed at 19°C, 27°C and 34°C. To investigate the influence of pH, an assay solution pH range between 4 and 9 was used.

Communoprecipitation assay

Agrobacterium strains carrying the pWM-HA-CYN and pWM-FlagCYN constructs were cultured in LB medium at 28°C until OD₆₀₀ >0.8. The cells were harvested and resuspended in infiltration medium (10 mM MgCl₂, 10 mM MES pH 5.7 and 20 μM acetoxyringerone) to OD₆₀₀ 0.8. Equal volumes of cell suspensions for the two strains were mixed and injected into leaves of N. benthamiana. After transient expression for 24–48 h, the leaves were harvested and ground to a powder in liquid nitrogen. The powder was thawed in two volumes of immunoprecipitation (IP) buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, and 1× protease complete inhibitor cocktail; Roche) and centrifuged at 12,000 x g for 10 min at 4°C. After centrifugation, 1 ml of supernatant was incubated with 50 μl of protein G Sepharose-4 fast flow beads (Amersham) and 0.5 µg of the indicated monoclonal antibody for 3 h at 4°C. After incubation, the immunocomplexes were washed four times with 1 ml IP buffer. The pellet samples were separated by SDS-PAGE and analysed by western blotting. The following antibodies were used: Mouse anti-FLAG antibody (clone M2, F3165, Sigma), Mouse anti-HA antibody (clone HA-7, H9658, Sigma), Goat anti-Mouse IgG (H+L) (Alkaline phosphatase conjugate, S3721, Promega) and Native mouse IgG (401111, Merek).

Identification of T-DNA Insertion and Plant Transformation

The AtCYN mutant plants were genotyped by PCR using the specific primers LBa1, Lp1, LP2, RP1, RP2 and RP3 (Table 3) designed with SALK (http://signal.salk.edu/tndaprimers.html). Accession numbers of the Atcyn mutant lines was listed in Table 4. The floral dip method [53] was used to transform the pWM-CYN constructs into cyn-7 mutant plants.

Northern blotting and quantitative RT-PCR analysis

Total RNA isolation and reverse transcription were performed as described above. The AtCYN segment was amplified using the primer pair AtCYN 5′/3′, and labelled with α-³²P-dCTP using the Prime-a-Gene Labeling System (Promega). Hybridization was performed in 50% formamide, 5× SSC, 5× Denhardt’s solution and 1% SDS with 100 mg/ml sperm DNA at 42°C. After overnight incubation, the blot membranes were washed twice in 2× SSC and 0.1% SDS for 10 min at room temperature and three times in 0.2× SSC and 0.1% SDS for 30 min at 42°C. Autoradiography of the blot membranes was carried out using the Cyclone phosphor imaging system (Packard Instruments).

Real-time PCR was performed using the SYBR® Green PCR Master Mix (Toyobo) with a DNA Engine Opticon TM 2 system (Bio-Rad). Each reaction was run in three technical replicate with at least three independent biological replicates. The primers used to amplify AtCYN were AtCYN P5/P6 and those used to amplify the internal control AtEIF4A were AtEIF4AF/R [54](Table 3). Transprimers levels of AtCYN (RE) were calculated using the following equation: RE = (E AtCYN / E AtEIF4A / E AtCYN(control-sample) / E AtEIF4A(control-sample)).

Author Contributions

Conceived and designed the experiments: DQ LJ YL. Performed the experiments: DQ LJ LL. Contributed reagents/materials/analysis tools: DQ. Collected data: DQ LJ LL. Analysis and interpretation of data: DQ LJ LL. Drafted manuscript: DQ LJ LL. Revised manuscript: DQ LJ LL. Final approval of the manuscript: DQ LJ LL.

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