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Overexpression of Cyanase in *Chlamydomonas reinhardtii*: a Promising Approach for Biodegradation of Cyanate in Aquatic Systems

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Abstract Cyanate and its derivatives are considered as highly dangerous materials that threaten human health and environment. Cyanate arises from both natural resources and anthropogenic activities including various chemical industries, herbicide production, and mining wastewater. Despite its toxicity, cyanate is considered as an important nitrogen (N) source in marine ecosystems. Cyanase (CYN) catalyzes the decomposition of cyanate into CO_2 and NH_3 in a bicarbonate-dependent reaction. In marine cyanobacteria, endogenous cyanases participate in detoxification of low concentrations of cyanate. However, this cyanate biodegradation system is seemingly inconvenient especially at contaminated sites due to high cyanate concentrations. In the current study, we have transferred the activity of the cyanobacterial enzyme cyanase into the micro-alga, *Chlamydomonas reinhardtii*, via *Agrobacterium tumefaciens*–mediated transformation method. The recombinant cyanase enzyme was shown to be active in transgenic *C. reinhardtii* lines. When variable concentrations of cyanate (up to 30 mM) is applied to growth medium, transgenic lines showed higher rate of NH_3 release, reduced loss of pigmentation symptoms, decreased levels of induced antioxidant enzymes, and low percentage of growth retardation compared to wild-type controls. Results of this study provide an effective eco-friendly phytoremediation system for cyanate detoxification using micro-algae compared to previously reported plant systems.

Keywords Cyanate · Phytoremediation · Transgenic *Chlamydomonas reinhardtii* · Synthetic biology

1 Introduction

Many researchers and research groups are now working on projects focusing on developing new technologies for the treatment of industrial and environmental hazardous wastes. Using biological treatment, systems received particular attention by many research groups. Aerobic and anaerobic microbial systems were applied

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successfully in the treatment and removal of such hazardous wastes including organic and inorganic compounds and metals (Mudder et al. 2001). Cyanide and its oxidation product cyanate are dangerous toxic chemicals produced through human activities and industries comprising ore leaching, electroplating, steal production, plastics, and synthetic fibers (Hamel 2011). Cyanate and its derivatives are being widely used for the manufacturing of a broad class of herbicides and in the synthesis of polymers (Koshiishi et al. 1997). Spontaneous photo-oxidation of cyanide as well as cyanide containing wastes are major sources for the release of the toxic cyanate into the environment (Malhotra et al. 2005; Mekuto et al. 2016). Moreover, cyanate is released into the environment through the biological breakdown of various metabolites such as urea and carbamoylphosphate (Guillotonm and Karst 1987). Chemical treatment methods such as oxidation or chlorination reactions are commonly applied for the detoxification of cyanate-containing compounds (Akcil and Mudder 2003). However, These chemical methods have some limitations such as high costs and production of hazardous byproducts (Srivastava and Muni 2010). Bioremediation systems based on the usage of plants or microorganisms are eco-friendly and more affordable alternatives (Akcil and Mudder 2003). In this regard, microbial degradation systems are probably inconvenient because of the accumulation of toxic metabolites and/or overloading the system with high amounts of the pollutant. This can negatively affect microbial growth and in turn the overall bioremediation process (Ebbs 2004). Phytoremediation using algal-based systems and/or vascular plant systems might become preferable alternatives for removal of cyanate-containing compounds and cyanide contaminants (Taebi et al. 2008).

Cyanase (EC 4.2.1.104) is able to degrade cyanate into CO_2 and NH_3 in the presence of bicarbonate (Johnson and Anderson 1987). The enzyme was first identified and fully characterized in *Escherichia coli* (Anderson and Little 1986; Taussig 1960; Walsh et al. 2000). Other Gram-positive bacteria and fungi were also shown to contain cyanase (Butryn et al. 2015; Kamennaya and Post 2010). Endogenous cyanase activity enables some bacteria to use cyanate as sole nitrogen and carbon source (Luque-Almagro et al. 2008; Taussig 1960). The enzyme was also isolated and characterized from cyanobacteria (Kamennaya et al. 2008; Voigt et al. 2014) and plants (Aichi et al. 1998; Qian et al. 2011). In living organisms, cyanases are important for degradation and/or removal of the toxic cyanide and cyanate compounds affecting their growth (Ebbs 2004). Moreover, cyanases are effectively involved in different biochemical and physiological pathways in plants. Application of KCNO to *Arabidopsis thaliana* cyanase knock-out mutants causes inhibition of seed germination and seedling development. On contrary, transgenic *Arabidopsis thaliana* plants overexpressing *Oryza sativa* (*Os*CYN) or *A. thaliana* (*At*CYN) cyanases showed better growth performance and increased resistance under cyanate stress (Qian et al. 2011). Recently, overexpression of cyanobacterial cyanase in *A. thaliana* enable transgenic plants to tolerate higher levels of cyanate, relative to wild type plants (Kebeish and Al-Zoubi 2017). However, cyanate biodegradation using transgenic plants is seemingly limited to soil ecosystems and cannot therefore be applied to water ecosystems.

Chlamydomonas reinhardtii has attracted more attention as a model for studying biological systems because this organism is the most biologically characterized (Rosales-Mendoza et al. 2012). Research into recombinant protein production such as expression of enzymes, proteins, human growth factors, antibodies, and vaccine in *Chlamydomonas reinhardtii* has attracted increasing attention (Rasala et al. 2010; Rosales-Mendoza et al. 2012). The efficacy of introducing cyanobacterial cyanase into micro-algae for cyanate remediation purposes has not been implemented so far. In the present study, the cyanobacterial cyanase gene (CYN, gi16329170) was therefore genetically cloned and transferred into *C. reinhardtii* nuclear genome. Transgenic *C. reinhardtii* lines showed enhanced tolerance to cyanate (up to 30 mM) compared to wild types. The biochemical response of transgenic micro-algae under cyanate stress has been studied in vivo. Results of the present study provide effective eco-friendly solutions for CNO^- remediation in aquatic systems.

2 Materials and Methods

2.1 *Chlamydomonas reinhardtii* Culture Conditions

The test organism, *Chlamydomonas reinhardtii* strains CC-124 (mt^-), was kindly obtained from Prof. Dr. Mohammed Ismaeil (Botany Department, Faculty of Science, Mansoura University, Egypt). *C. reinharditii* was aseptically grown in Tris Acetate Phosphate (TAP)

medium pH 7.4 (Gorman and Levine 1965). For selection of transformed *C. reinhardtii* colonies, solid TAP medium supplemented with 1.5% (*w*/*v*) agar and kanamycin (50 μg/ml) was used. The algal cultures were incubated in growth chamber at 25 ± 2 °C under long day conditions (16 h light/8 h dark) and kept under light intensity of 80 μmol m^{-2} s^{-1} with continuous shaking (75 rpm) in case of liquid cultures.

2.2 Gene Cloning and Plasmid Constructs

Cyanase gene (CYN gi16329170) was PCR-amplified using genomic DNA of *Synechoccous elongatus PCC6803* as template (kindly obtained from Botany Department, Faculty of Science, Cairo University, Giza, Egypt). A forward primer with extension for *Nco*I site (5′- ATGGCCATGGCTGG CACTGAAATTTC-3′) and a reverse primer (5′-GTCACTCGAGCCATTTCTTGTAGGGTAA-3′) with extensions for *Xba*I site were used. Amplified CYN fragments were genetically cloned into the binary expression vector pTRAK (gi13508478). The resulted CYN gene expression cassette (Fig. 1) was flanked by 5′-UTR of the Cab22L tobacco leader peptide (TL), 3′-UTR of CaMV 35S (pA35S), and the scaffold attachment region (SAR gi3522871). The pPCV002 nptII gene was used for kanamycin selection of transgenic *C. reinhardtii* (Koncz and Schell 1986). CYN gene expression is transgenic lines is controlled by CaMV-35S promoter (Reichel et al. 1996).

2.3 Transformation and Generation of Transgenic *C. reinhardtii* Lines

pTRAK-CYN construct was transformed into *C. reinhardtii* by the *Agrobacterium tumefaciens* (GV3101)-mediated co-cultivation transformation protocol as previously described (Kumar et al. 2004). single colony of wild type *C. reinhardtii* was inoculated into liquid TAP medium and was grown till reaching log phase. Cells were then plated on solid TAP medium and incubated for 48 h under continuous light until forming a lawn of cells. An *Agrobacterium* culture transformed with pTRAK-CYN plasmid was grown in liquid LB medium containing appropriate antibiotics (25 mg/l rifampicin and 50 mg/l kanamycin) at 28 °C till OD_{600} reaches 0.6. *A. tumefaciens* cells were then spun down (4000 ×*g* for 10 min at 4 °C) and resuspended in 250 μl liquid TAP medium containing 100 μM acetosyringone. The bacterial suspension was then co-cultivated with *C. reinhardtii* cells grown on the agar plates for 2 h at 28 °C followed by overnight incubation at 25 °C in dark. *C. reinhardtii* cells were harvested and washed twice with liquid TAP medium supplemented with 0.5 g/l cefotaxime (10 min each) in order to remove the remaining *Agrobacterium*. For selection of the transformed *C. reinhardtii* cells, the washed cells were cultivated on solid TAP agar plates containing 0.5 g/l cefotaxime and 50 μg/ml kanamycin and incubated at 25 °C in growth chamber for 8 days until the appearance of the transformed colonies (Fig. 2a). The presence of CYN transgene in the selected *Chlamydomonas* colonies was confirmed by colony PCR test using CYN gene–specific primers (2.2). Three independent transgenic *C. reinhardtii* lines were selected for further molecular and biochemical analyses. Presence of CYN transgene in transformed *C. reinhardtii* lines were confirmed by PCR test using genomic DNA isolated from transgenic lines as template prior to each assay.

2.4 qRT-PCR Analysis

Quantitative RT-PCR was performed as previously described (Kebeish and Al-Zoubi 2017). RNA was extracted from *C. reinhardtii* cells following the BCP

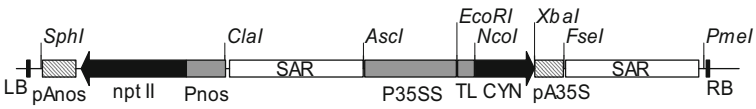

Fig. 1 Structure of cyanobacterial cyanase (CYN) gene expression cassette. *Synechococcus elongatus PCC 6803* cyanase gene coding sequence (CYN, gi16329170, E.C.4.2.1.104) was cloned into the binary plant expression vector pTRA-K (gi13508478) in between *NcoI* and *XbaI* sites. CYN gene cassette is flanked by Scaffold attachment regions (SAR) of the tobacco RB7 gene (gi3522871). CYN gene expression is under the control of the constitutive CaM promoter (p35SS), 5′ UTR of the Cab22L tobacco leader peptide (TL), and 3′ UTR of CaMV 35S (pA35S). LB/RB: Left and right border sequences of Nopaline-Ti-plasmids pTiT37. pAnos: Polyadenylation signal of Nopaline synthetase gene from *A. tumefaciens.* NptII: Neomycin phosphotransferase type II that confers resistance to kanamycin. pnos: Promoter of nopaline synthase gene from *A. tumefaciens*

(1-bromo-3-chlorpropane) protocol of Chomczynski and Mackey (Chomczynski and Mackey 1995). The protocol described by Niessen et al. (2007) was applied for the synthesis of first-strand cDNA. qRT-PCR analysis was performed on An ABI PRISM1 7300 (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions. SYBR Green Reagents (Karlsruhe, Germany) were used for PCR amplifications. Oligonucleotides were purchased from Intron Biotechnology Inc. (Kyungki-Do, South Korea). For CYN transcripts, primers 5′-GGG AAT CAC GTT TGC TGA TTT-3′ and 5′-AAG TTT CTC CGC CTC ATC AA-3′ were used. Primers 5′-GCG ATG TGG ACA TCC GCA AG-3′ and 5′-GGG CCG TGA TCT CCT TGC TC-3′ were used for detection of ACTIN transcripts. In the reaction mixtures, final primer concentration was adjusted to be 200 nM. 10 min primary denaturation at 95 °C, followed by 40 cycles (15 s denaturation at 95 °C and 60 °C combined annealing and extension for 1 min) was applied as a PCR program for amplification of both CYN and ACTIN.

2.5 Growth Assay of Transgenic *C. reinhardtii* under Cyanate Stress

Three independent *C. reinhardtii* lines transgenic for cyanase gene in addition to wild type were used for evaluating the efficient detoxification of cyanate from the culture medium. Transgenic and wild-type *C. reinhardtii* ($\sim 5 \times 10^3$ cells) were inoculated into liquid TAP medium supplemented with 0, 5, 10, 15, 20, and 30 mM KCNO. After that cells were allowed to grow at long day growth conditions with continuous shaking. Cell densities at 665 nm (Robert 1979) were recorded each 24 h for 15 days. A growth response curve was generated and mid log phase of growth was observed after 7 days. The experiment was then repeated in trice and cell density for each genotype at all the applied KCNO concentrations were recorded after 7 days of growth where the percentage of growth reduction as a result of KCNO application was calculated based on the following equation: % reduction in growth = [(cell density of *C. reinhardtii* without KCNO − cell density at the

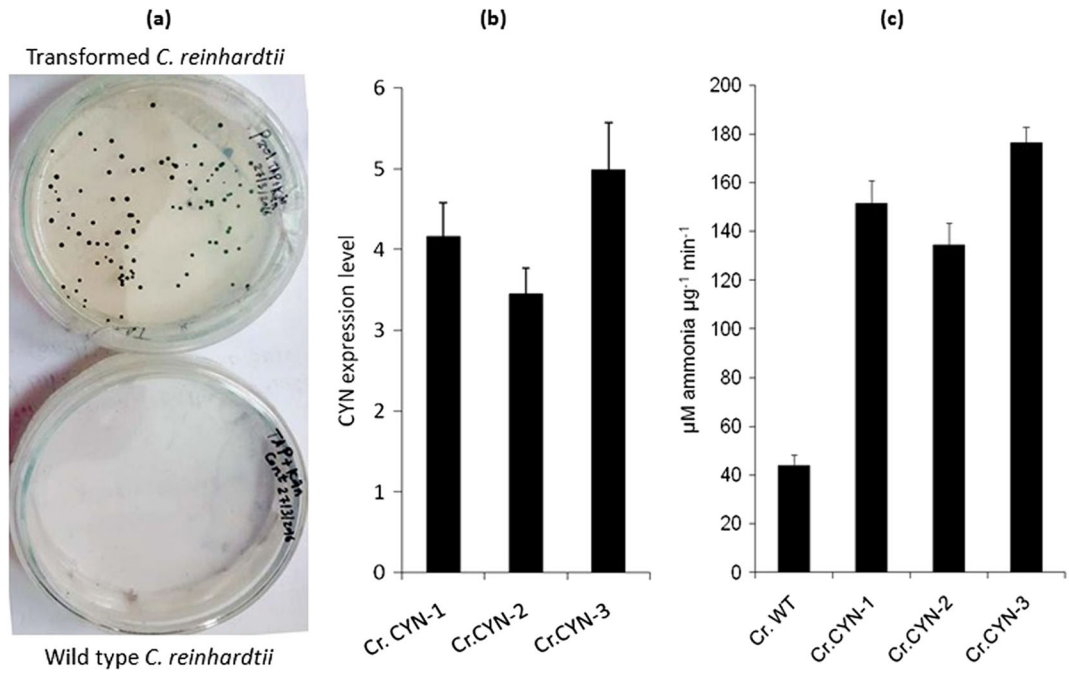

Fig. 2 Selection of transformed colonies of *C. reinhardtii*, qRT-PCR analysis of CYN transgene expression and cyanase enzymatic activity in transgenic lines of *C. reinhardtii*. **a** Photograph representing selection of the transformed colonies of *C. reinhardtii* and wild-type cells on solid TAP medium supplemented with kanamycin. **b** Results of qRT-PCR analysis of CYN transcript accumulation in three chosen transgenic colonies of *C. reinhardtii*. **c** Activity of cyanase enzyme measured from transgenic C. *reinhardtii* lines (Cr.CYN-1 to 3). Each data point is based on at least three independent measurements. Error bars indicate SE

desired KCNO concentration) / (cell density of *C. reinhardtii* without KCNO)] × 100.

2.6 Ammonia Release and Pigment Content Measurements

Ammonia released from transgenic *C. reinhardtii* at different concentrations of potassium cyanate was carried out according to the method described by Rasco-Gaunt et al. (Rasco-Gaunt et al. 1999) with modifications. Wild-type and transgenic *C. reinhardtii* cells were allowed to grow in liquid TAP medium for 7 days. Algal tissues were harvested and 0.05 g from each genotype were transferred into 2-ml micro tubes containing 1 ml of incubation medium [potassium phosphate pH 5.8 (50 mM), sucrose (2%), Tween 20 (1%), 2,4-dichlorophenoxy acetic acid (0.1 mg l^{-1}), and phosphinotricin (25 mg/l)] supplemented with 0, 5, 10, 15, 20, and 30 mM KCNO. As blanks, three reaction micro tubes containing incubation medium without KCNO were used. The test samples were incubated for 16 h at 25 °C under 80 μmol m^{-2} s^{-1} light. After centrifugation (13,000 rpm/5 min), 100 μl from each sample were transferred into a new 1.5-ml reaction tube containing 0.5 ml of reagent-I (0.4 mM sodium nitroprusside, 25 g/l sodium tartrate, 0.085 M trisodium citrate, and 0.21 M sodium salicylate). Samples were mixed and 500 μl of reagent-II (2.3 mM sodium dichloroisocyanurate, 0.75 M NaOH) was added. The reaction mixtures were then left in the dark at 37 °C followed by incubation at room temperature for 15 min. Ammonium ions in the test samples were quantified by measuring the absorbance at 655 nm compared to standards of ammonium chloride.

Pigments, *Chl. a*, *Chl. b*, and carotenoids were extracted from wild-type and transgenic *C. reinhardtii* cells grown for 5 days in liquid TAP medium after application of different concentrations of KCNO (0, 5, 10, 15, 20, 30 mM) with 80% acetone. After homogenization and vigorous shaking, mixtures were centrifuged at 30.000 g for 10 min. Absorbance of the supernatants was measured at 664, 647, 630, and 452 nm. Pigment contents were calculated as described by Metzner et al. (1965) and Jeffrey and Humphrey (1975).

2.7 Protein Extraction and Enzymatic Assays

For total protein extraction from wild-type and transgenic *C. reinhardtii* cells, 100 mg fresh algal tissues was collected and directly frozen in liquid nitrogen. Frozen pellets were then homogenized and 500 μl of extraction buffer (50 mM sodium phosphate buffer (pH 7.4) containing 0.1% Triton X-100, 10 mM EDTA, 2% PVP, and 20% glycerol) were added. The homogenate was centrifuged twice at 30.000 ×*g* for 20 min at 4 °C. Supernatants were then collected and protein concentration was determined using Bradford method (Bradford 1976). These protein extracts were used for cyanase (CYN), catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) enzymatic assays based on the assay conditions.

Cyanase activity was measured in vitro from wild-type and transgenic *C. reinhardtii* cells following the protocol described by Anderson (P.M Anderson 1980). The reaction mixture composed of 50 mM potassium phosphate buffer (pH 7.7), 3 mM $NaHCO_3$, and 2 mM KCNO. The reaction was started by addition of protein extract and stopped by addition of equal volumes of Nessler's reagent after 1–10 min at 26 °C. Ammonium chloride dilution series were used for generating a standard curve. The amount of released ammonia (μmol) was estimated by measuring the optical density at 420 nm 10 min after addition of Nessler's reagent compared to ammonium chloride standards. Cyanase activity was expressed as μmol ammonia released/mg protein/min.

For determination of antioxidant enzyme activities for wild-type and transgenic *C. reinhardtii* lines under cyanate stress, total proteins were extracted from 100 mg *C. reinhardtii* cells harvested after 5 days growth in liquid TAP medium supplemented with 0, 5, 10, 15, 20, and 30 mM KCNO. Extracted proteins from each genotype were used for quantification of catalase (Kar and Mishra 1976), peroxidase (Racusen and Foote 1965), and superoxide dismutase (Beauchamp and Fridovich 1971; Xu et al. 2008) activities at all potassium cyanate concentrations.

2.8 Statistical Analysis

Data were represented as mean ± standard error (SE) of at least three independent measurements. Student's *t* test using Excel software (Microsoft Corporation, USA) was applied to determine significance.

3 Results and Discussion

3.1 Gene Cloning and Generation of Transgenic *C. reinhardti*i Lines

The target of the present study was to test whether overexpression of the cyanobacterial cyanase (CYN, gi16329170) could support the growth and cyanate remediation capacity of *C. reinhardtii* or not. Among species, different pathways for cyanase-dependent cyanate metabolism have been documented. In bacteria, cyanase was shown to have an active role in the production of NH_3 as an alternative source for nitrogen from cyanate (P.M. Anderson et al. 1990). Products of cyanase activity in photosynthetic cyanobacteria are used as carbon and nitrogen sources (Espie et al. 2007). The gene coding for cyanase has been identified, characterized, and genetically expressed in *Arabidopsis* (Aichi et al. 1998; Qian et al. 2011). Cyanase (*cynS)* was also characterized in *Synechocystis* sp. strain PCC7942, PCC6803, and PCC6301 (Harano et al. 1997). In the current study, cyanobacterial gene, CYN gi16329170, was used for the transformation of *Chlamydomonas reinhardtii* in order to evaluate the efficacy of this cyanobacterial cyanase in promoting cyanate remediation capacity of *C. reinhardtii* cells. Wild-type *C. reinhardtii* was transformed with the binary expression construct, pTRAK-CYN (Fig. 1) via *Agrobacterium tumefaciens*–mediated co-cultivation method (Kumar et al. 2004).

Transgenic *Chlamydomonas* lines were selected on solid TAP medium supplemented with kanamycin antibiotic (Fig. 2a). Three independent lines of transgenic *Chlamydomonas reinhardtii* named Cr.CYN-1, Cr.CYN-2, and Cr.CYN-3 were randomly selected and used for further molecular and biochemical analyses.

3.2 RT-PCR and Cyanase Enzymatic Assays

CYN gene mRNA transcripts were initially measured in CYN transgenic lines by qRT-PCR (Fig. 2b). High levels of CYN transcripts were observed for all the tested CYN transgenic *C. reinhardtii* lines compared to ACTIN transcripts. Transgenic line Cr.CYN-3 shows relatively higher levels of cyanase transgene expression followed by Cr.CYN-1 then Cr.CYN-2 lines. Variable endogenous promoters controlling gene expression in *C. reinhardtii* have been reported. The small subunit of the ribulose bisphosphate carboxylase, RbcS2 promoter was the most widely used for induction of transgene expression in *C. reinhardtii* (Cerutti et al. 1997). However, other constitutive promoters as PsaD, TubA1, Hsp70A, AtpC, and β-tub (Rosales-Mendoza et al. 2012) were shown to induce high transgene expression in chimeric constructs used for *C. reinhardtii* transformation. Moreover, Cauliflower mosaic virus 35S promoter (CaMV 35S) has also been shown to induce high gene expression in this micro-alga (Tang et al. 1995). It was previously reported that high expression of transgenes in the model organism *C. reinhardtii* could be enhanced when using genes comprising high GC content (Heitzer et al. 2007). Since GC content of the introduced cyanobacterial cyanase gene is approximately 47%, the observed expression levels of this transgene are therefore reasonable (Fig. 2b). However, codon usage adaptation of the introduced transgene is necessary for this micro-alga to induce higher expression levels.

To test the functionality of the expressed cyanase in transgenic lines, the activity of cyanase transgene was also tested on protein level in extracts of WT and transgenic *C. reinhardtii* lines in vitro. The results of cyanase activity assay are shown in Fig. 2c. Threefold to fourfold increase in the activity of cyanase was obvious for transgenic lines compared to WT controls. CYN-1 and CYN-3 lines show more activity for cyanase when compared to line CYN-2 (Fig. 2c). The background activity observed for WT control samples may be due to the action of endogenous cyanases and other nitrogen assimilating enzymes (Aichi et al. 1998; Anderson and Little 1986). It can be therefore concluded that the prokaryotic cyanase is active in transgenic *C. reinhardtii* lines.

3.3 Ammonia Release Assay from Transgenic *C. reinhardtii*

Cyanase converts cyanate into CO_2 and NH_3 in the presence of bicarbonate. Therefore, the amount of ammonia released from wild-type and transgenic *C. reinhardtii* lines in the presence of variable concentrations of cyanate was measured. This assay relies on suppression of ammonia fixation/re-fixation in the GS2/Fd-GOGAT cycle by phosphinotricin (Rasco-Gaunt et al. 1999). Wild-type and transgenic *C. reinhardtii* fresh cells were incubated in medium containing 2 mM $NaHCO_3$ and variable concentrations of KCNO (i.e., 0.0, 5, 10, 15, 20, and 30 mM). Results of this assay are shown in Fig. 3. In the

absence of KCNO, NH_3 release was not significantly affected in both transgenic and wild-type control samples. A gradual decrease in the amount of NH_3 was observed for wild-type *C. reinhardtii* by increasing KCNO concentrations. On the contrary, significant KCNO concentration-dependent ($p \leq 0.001$) increases in the amount of NH_3 released was observed for all the three transgenic lines compared to the corresponding wild-type controls. Again, line Cr.CYN-3 was superior compared to Cr.CYN-2 and Cr.CYN-1 lines. Reduction of ammonia release observed for wild type control samples may be due to the limited capacity of the endogenous cyanase to overcome the excess cyanate applied to the reaction mixture (Qian et al. 2011). In microbes, induction of cyanase gene transcription can be achieved through the application of exogenous cyanate (Luque-Almagro et al. 2008). However, induction of cyanase gene transcription in *Arabidopsis thaliana* and *Oryza sativa* was only observed under salt stress but not cyanate stress (Askari et al. 2006; Qian et al. 2011). Results of NH_3 release assay indicate that CYN transgenic *C. reinhardtii* is able to produce more ammonia from exogenously applied cyanate and this, in turn, proves the functionality of the cyanobacterial cyanase enzyme in the green micro-alga, *Chlamydomonas reinhardtii* concerning cyanate remediation. This supports the significance of using CYN transgenic lines in cyanate detoxification.

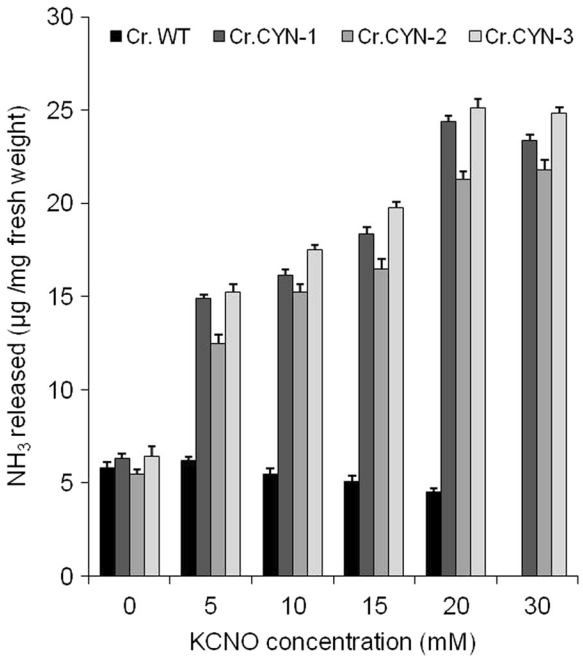

Fig. 3 Ammonia release assay from wild-type and CYN transgenic *C. reinhardtii* under KCNO stress. Amount of NH_3 released from wild type (Cr. WT) and three cyanase transgenic *C. reinhardtii* lines (Cr.CYN1-3) was measured from *Chlamydomonas reinhardtii* cells incubated with different concentrations of KCNO (0.0, 5, 10, 15, 20, and 30 mM) for 4 h. Each data point represents the average of at least three independent measurements ± SE. *P* value < 0.001 for transgenic lines compared to wild type.

3.4 Growth Assays of Transgenic *C. reinhardtii* Under Cyanate Stress

In order to evaluate the efficacy of the cyanobacterial transgene cyanase in cyanate detoxification, a growth assay was performed using CYN transgenic *C. reinhardtii* lines. Similar amounts of Cr.CYN-1, Cr.CYN-2, Cr.CYN-3, and wild-type cells were inoculated into liquid TAP medium supplemented with 0.0, 5, 10, 15, 20, and 30 mM KCNO and grown for 7 days. Algal cell densities were then measured at 665 nm and the percentage of growth reduction was calculated. The results of this assay are shown in Fig. 4 and Table 1. A concentration-dependent inhibition of *Chlamydomonas* growth was obvious for wild type as well as CYN transgenic lines. However, growth of transgenic lines is highly significant compared to wild type at all potassium cyanate concentrations applied for this assay (Fig. 4). A sharp decrease in growth of wild-type cells was also remarkable at KCNO concentrations above 10 mM (59% and 88% reduction in growth at 15 and 20 mM KCNO, respectively) leading to a complete cell death at 30 mM KCNO (Table 1). On contrary, transgenic lines showed better growth performance and a decreased percentage of growth reduction compared to wild-type cells and growth of Cr.CYN-1 was relatively higher compared to Cr.CYN-2 and Cr.CYN-3 lines (Table 1). These growth assay results under cyanate stress indicate that expression of the cyanobacterial cyanase transgene in *Chlamydomonas* enhances the ability of this micro-alga to metabolize large amounts of the toxic cyanate from the culture medium (i.e., up to KCNO concentration of 20 mM) and consequently used its degradation products (i.e., CO_2 and NH_3) for supporting algal growth and biomass production. The increased reduction in growth of wild type starting from concentration of 10 mM KCNO may be attributed to the inefficient capacity of

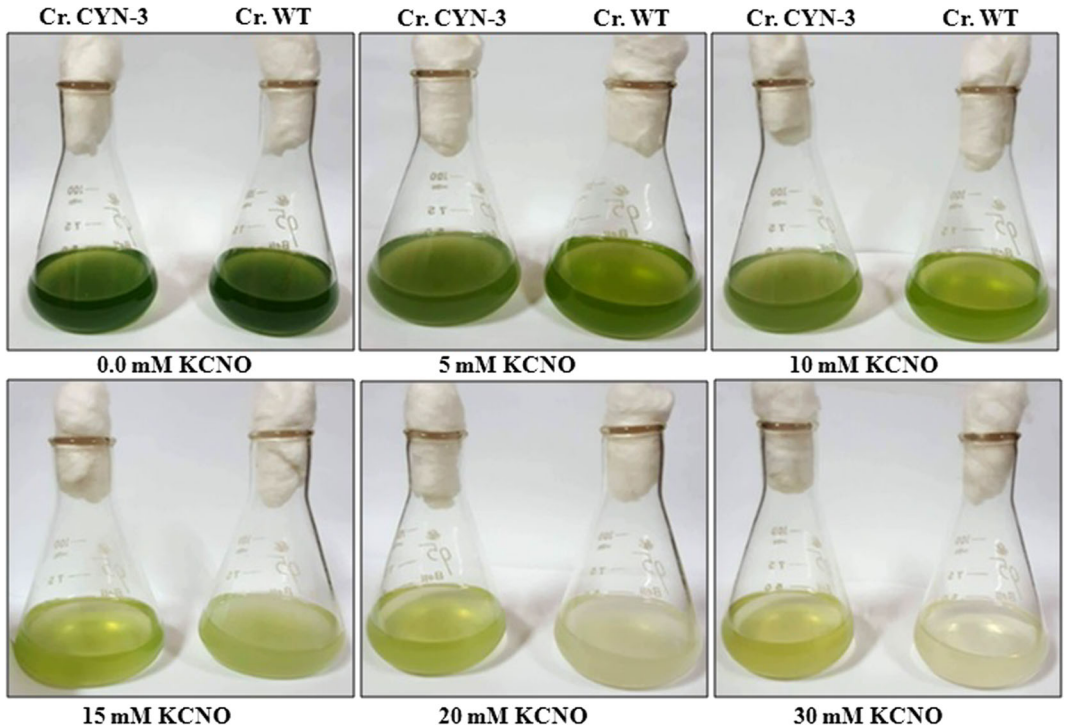

Fig. 4 Effect of different KCNO concentrations on growth of wild-type and CYN transgenic *C. reinhardtii*. Photographs representing growth of wild-type and transgenic *C. reinhardtii* after 7 days in TAP medium containing different concentrations of KCNO.

the endogenous cyanate metabolizing enzymes to remove the applied cyanate from the growth medium (Qian et al. 2011; Askari et al. 2006). It is important to compare the current growth assay results obtained for CYN transgenic *C. reinhardtii* with our previously reported results for CYN transgenic *A. thaliana* plants (Kebeish and Al-Zoubi 2017). A similar growth retardation phenomenon was observed for wild-type *A. thaliana* plants when cyanate concentration of 2.5 mM was applied to the foliar parts of the plant. Additionally, 0.4 mM cyanate in the culture medium caused a total inhibition of root hair formation in wild-type *Arabidopsis* plants. However, in both growth assays, expression of the cyanobacterial enzyme in *A. thaliana* plants enhance plant resistance to the exogenously applied cyanate up to 1.2 mM. Indeed, the enhanced growth of CYN transgenic *C. reinhardtii* at higher cyanate concentrations (i.e., 15, 20, and 30 mM KCNO) compared to wild-type cells support the usage of CYN transgenic *C. reinhardtii* as an efficient bio-system for detoxification of the harmful cyanate from the environment, especially from the contaminated water sites.

3.5 Effect of KCNO on Pigment Contents in Wild-Type and Transgenic *C. reinhardtii* Cells

Initial growth assays with wild-type *C. reinhardtii* showed that cyanate application in growth media leads to growth retardation accompanied by a loss of pigmentation symptoms. Therefore, chlorophyll and carotenoid pigments were measured from Cr.CYN transgenic lines and compared to wild-type controls under KCNO stress. Results of this assay are shown in Table 2. All the measured values for pigment contents were significantly higher in cyanase overexpressors compared to wild-type controls under the assay conditions. The observed values for *chl.a*, *chl.b*, and carotenoids in Cr.CYN-1, Cr.CYN-2, and Cr.CYN-3 transgenic lines at KCNO concentration of 20 mM reached about 1.9-, 1.8-, and 2.0-fold, respectively, compared to their wild-type counterparts. It was also observed that 30 mM KCNO cause a complete death of wild-type *C. reinhardtii* cells. However, transgenic *C. reinhardtii* can survive this high concentration of cyanate with weak signs of growth retardation symptoms. The decrease in pigment contents observed for wild-type *C. reinhardtii* may be due to

Table 1 Percentage of reduction in wild type and CYN transgenic *C. reinhardtii* growth after incubation in different concentration of KCNO

	% reduction in *C. reinhardtii* cell density				
KCNO (mM)	5	10	15	20	30
Cr.WT	16.7 ± 2.3	34.1 ± 2.5	59.1 ± 3.4	88.7 ± 4.7	100
Cr.CYN-1	01.0 ± 0.1	05.8 ± 0.4	16.8 ± 1.2	25.2 ± 2.4	61.1 ± 2.3
Cr.CYN-2	01.9 ± 0.2	08.0 ± 0.8	19.9 ± 1.3	34.1 ± 2.6	65.1 ± 4.8
Cr.CYN-3	01.1 ± 0.3	05.5 ± 0.3	17.4 ± 1.8	27.4 ± 2.7	62.2 ± 2.6

Percentage of reduction in cell density measured from wild type and three independent transgenic *C. reinhardtii* treated with 5, 10, 15, 20, 30 mM KCNO after 7 days of growth. Data are means of three independent preparations from each genotype. All values recorded for transgenic lines are statistically significant compared to wild-type control *C. reinhardtii* cells. ($n = 3 \pm$ SE). p value < 0.001

inhibition of vital enzymes contributing to metabolic processes related to pigment biosynthesis and viability by the applied cyanate (Samuilov et al. 2006; Solomonson 1981). The mitochondrial cytochrome c oxidase that is actively involved in the respiratory electron transport chain was reported to be inhibited by cyanide and its oxidative product cyanate (Way 1984). Ribulose-1,5-bisphosphate carboxylase/oxygenase (Wishnik and Lane 1969) and stress-relieving enzymes involved in ROS scavenging mechanisms have been shown to be inhibited by cyanides (Karuppanapandian et al. 2011). Additionally, cyanide and cyanate are also able to inactivate important metalloenzymes involved in many biological processes such as respiration, carbon, and nitrate assimilation (Leavesley et al. 2008; Solomonson 1981). The observed results for pigment contents in Cr.CYN transgenic lines (Table 2) are in accordance with our previous results reported for *A. thaliana* plants transgenic for the cyanobacterial enzyme cyanase (Kebeish and Al-Zoubi 2017) or for the bacterial enzyme cyanidase (Kebeish et al. 2015). Transgenic *A. thaliana* plants overexpressing the cyanase or

Table 2 Effect of different concentrations of KCNO on pigment contents in wild type and transgenic *C. reinhardtii* cells

KCNO (mM)	0.00	5	10	15	20	30
Chl.a (μg/mg fresh weight)						
Cr.WT	19.8 ± 0.9	16.1 ± 0.6	14.1 ± 0.6	12.1 ± 0.9	9.0 ± 0.7	n.d
Cr.CYN-1	20.5 ± 0.8	20.5 ± 0.9	19.1 ± 0.6	19.1 ± 0.5	17.3 ± 0.4	14.4 ± 0.3
Cr.CYN-2	21.3 ± 1.1	20.5 ± 1.2	18.5 ± 0.5	17.8 ± 1.3	16.5 ± 0.6	13.8 ± 0.8
Cr.CYN-3	20.9 ± 0.7	20.8 ± 0.5	20.5 ± 0.2	20.5 ± 0.8	18.5 ± 0.7	16.2 ± 0.6
Chl.b (μg/mg fresh weight)						
Cr.WT	30.5 ± 1.2	25.5 ± 0.9	23.9 ± 1.3	16.3 ± .06	8.9 ± 0.08	n.d
Cr.CYN-1	32.4 ± 1.3	31.6 ± 0.9	28.6 ± 0.7	28.0 ± 0.8	24.8 ± 1.4	23.0 ± 1.2
Cr.CYN-2	30.9 ± 1.2	30.0 ± 0.7	27.6 ± 1.1	26.2 ± 0.6	23.1 ± 0.5	20.8 ± 1.2
Cr.CYN-3	31.5 ± 0.8	31.6 ± 0.9	30.7 ± .04	28.8 ± 0.5	27.0 ± 0.8	25.1 ± 1.0
Carotenoids (μg/mg fresh weight)						
Cr.WT	17.8 ± 0.8	11.9 ± 0.3	11.5 ± 0.8	09.0 ± 0.3	07.8 ± 0.1	n.d
Cr.CYN-1	17.8 ± 0.4	16.4 ± 0.5	16.1 ± 0.2	15.8 ± 0.5	14.8 ± 0.2	09.5 ± 0.4
Cr.CYN-2	17.2 ± 0.5	15.8 ± 0.8	14.6 ± 0.4	14.3 ± 0.8	12.5 ± 0.9	08.3 ± 0.8
Cr.CYN-3	17.9 ± 0.4	17.6 ± 0.5	17.2 ± 0.7	16.5 ± 0.8	15.3 ± 0.3	10.5 ± 0.2

Chlorophyll a (*Chl.a*), Chlorophyll b (*Chl.b*), and carotenoids' contents measured from wild type and three independent transgenic *C. reinhardtii* treated with 0, 5, 10, 15, 20, 30 mM KCN. Data are means of three independent preparations from each genotype. Vertical bars represent standard error. All the measured values of transgenic lines are statistically significant when compared with wild-type control *C. reinhardtii* cells. ($n = 3 \pm$ SE). p value < 0.001

cyanidase showed significant increase in chlorophyll a, chlorophyll b, and carotenoids contents under cyanate or cyanide stress, respectively. The observed results in this assay indicate therefore that KCNO-dependent loss of pigmentation symptoms was ameliorated through the activity of the recombinant cyanobacterial enzyme cyanase in transgenic *C. reinhardtii* lines.

3.6 Effect of KCNO on Antioxidant Enzyme Activities in Wild-Type and Transgenic *C. reinhardti*i Cells

Algae and higher plants show variable biochemical responses to biotic and abiotic stress conditions. Among these biochemical responses is the production of antioxidant enzymes in order to reduce the endogenously generated reactive oxygen species (ROS) (Liu et al. 2018). Therefore, the antioxidant enzymes, catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) activities were measured in extracts of transgenic and wild-type lines (Table 3) at different concentrations of KCNO. Wild-type extracts show enhanced levels of antioxidant enzyme activities at all the applied cyanate concentrations in a relative concentration-dependent manner. However, significant reduction in antioxidant enzymes was recorded for CYN transgenic lines compared to their corresponding controls of wild type at both low and high concentrations of KCNO. At KCNO concentration of 20 mM, wild-type *C. reinhardtii* showed 3.4-fold increase for CAT, 2.4-fold increase for POD, and 5-fold increase for SOD compared to untreated cells. However, transgenic lines showed low induction of these enzymes (−31% catalase, −42% peroxidase, and −48% superoxide dismutase) compared to wild-type controls at the same KCNO concentration (i.e., 20 mM). Similar reduction in antioxidant enzyme activities was also observed for CYN transgenic lines at 5 mM, 10 mM, and 15 mM KCNO. Cr.CYN-3 line was superior when compared to the other two transgenic lines (Table 3). The observed reduction of CAT, POD, and SOD activities in transgenic *C. reinhardtii* lines can be due to the activity of the recombinant cyanobacterial enzyme cyanase in detoxification of the applied cyanate. SOD is considered as the first key player in the defense mechanism against oxidation as it converts O_2^- to H_2O_2 and O_2 (Bowler et al. 1992). Previous studies have shown significant increase in SOD activities under KCN stress in *Chlorella vulgaris* however at low concentrations (0.1–10 mg l^{-1}), hence SOD contributed to the removal of superoxides. On contrary, by increasing KCN concentration to 10 mg l^{-1}, a decline in SOD activity was observed (Liu et al. 2018). This may be due to the

Table 3 Effect of different KCNO concentrations on antioxidant enzyme activities in wild type and transgenic *C. reinhardtii* cells

KCNO (mM)	0.00	5	10	15	20	30
CAT (activity/mg protein/min)						
Cr.WT	1.4 ± 0.5	2.6 ± 0.2	3.1 ± 0.2	3.8 ± 0.6	4.8 ± 0.3	n.d
Cr.CYN-1	1.5 ± 0.2	2.1 ± 0.1*	2.4 ± 0.2**	2.8 ± 0.3**	3.0 ± 0.2***	3.8 ± 0.4***
Cr.CYN-2	1.6 ± 0.3	2.5 ± 0.2	2.6 ± 0.3*	2.9 ± 0.3**	3.2 ± 0.2***	3.7 ± 0.2***
Cr.CYN-3	1.4 ± 0.3	2.2 ± 0.3*	2.5 ± 0.3**	2.8 ± 0.3**	4.1 ± 0.2*	3.5 ± 0.4***
POD (activity/mg protein/min)						
Cr.WT	3.5 ± 0.3	4.7 ± 0.3	5.1 ± 0.3	6.9 ± 0.3	8.6 ± 0.3	n.d
Cr.CYN-1	3.1 ± 0.3	3.8 ± 0.3	4.7 ± 0.3*	4.9 ± 0.2**	5.1 ± 0.3***	6.8 ± 0.4***
Cr.CYN-2	3.2 ± 0.5	3.9 ± 0.3	4.5 ± 0.2*	5.0 ± 0.4*	5.5 ± 0.5***	7.2 ± 0.3***
Cr.CYN-3	3.3 ± 0.4	3.5 ± 0.3**	4.1 ± 0.3**	4.5 ± 0.3***	4.8 ± 0.2***	5.7 ± 0.2***
SOD (activity/mg protein/min)						
Cr.WT	1.5 ± 0.2	2.9 ± 0.1	4.8 ± 0.2	6.7 ± 0.2	7.6 ± 0.1	n.d
Cr.CYN-1	1.3 ± 0.2	2.0 ± 0.1**	2.8 ± 0.2***	3.4 ± 0.2***	3.9 ± 0.1***	5.5 ± 0.4***
Cr.CYN-2	1.4 ± 0.2	2.5 ± 0.2*	3.0 ± 0.3**	3.6 ± 0.3***	4.5 ± 0.3**	5.8 ± 0.4***
Cr.CYN-3	1.6 ± 0.3	2.2 ± 0.3**	2.8 ± 0.4***	3.1 ± 0.6***	3.5 ± 0.5***	4.8 ± 0.3***

Catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) activities measured from wild type and three independent transgenic *C. reinhardtii* treated with 0, 5, 10, 15, 20, 30 mM KCNO for 4 days. Data represent the average of three independent measurements from each genotype ± SE. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

inhibition of SOD activity by excess cyanide. Many reports have indicated that cyanide and perhaps cyanate inhibit antioxidant enzyme activities (Oracz et al. 2009; Siegien and Bogatek 2006). However, our recently published studies with *A. thaliana* expressing either cyanidase or cyanase (Kebeish et al. 2015; Kebeish and Al-Zoubi 2017) indicates the effective role of cyanide and/or cyanate-degrading enzymes in reducing the negative impact of the toxic cyanide compounds on the antioxidant machinery in transgenic plants through the efficient removal and/or degradation of the toxic factors. Therefore, the observed decrease in the activities of antioxidant enzyme in C. *reinhardtii* transgenic for CYN may reflect the efficient role of the recombinant cynobacterial cyanase in the removal of the applied cyanate from the surrounding growth medium. Taken together, the observed biochemical assay data in this study provide a strong evidence for the significance of this novel micro-algal–based detoxification system for cyanate removal from the environment.

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