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ORIGINAL ARTICLE

Chlamydomonas reinhardtii, a model system for functional validation of abiotic stress responsive genes

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Abstract Stress tolerance is a multigenic character and there are many stress responsive genes, which are stress specific. Although many of these have been cloned, their functional significance remains fragmentary. Hence it is important to identify the relevant stress genes involved in altering the metabolism for adaptation. Overexpression is one of the several approaches and *Chlamydomonas* is a suitable system to study the functional relevance of stress genes. Stress responses can only be assessed on prior exposure to sublethal induction stress. In this study the acclimation response of *Chlamydomonas* was assessed for different abiotic stresses using physiological screens like chlorophyll stability, membrane damage, cell viability, accumulation of free radicals, survival and recovery growth. We demonstrate that *Chlamydomonas* responds to diverse stresses and is a potential system to study the relevance of stress genes. The relevance of choline oxidase A (codA), a key enzyme in glycinebetaine biosynthesis, was examined by developing transformants expressing *codA* gene from *Arthrobacter globiformis*. Southern positive transformants showed enhanced accumulation of glycinebetaine. The transformants also showed enhanced growth under salinity, high light coupled with methylviologen-induced oxidative stress, high temperature and cold stress. However the transgenics were not tolerant to PEG-mediated simulated osmotic stress, LiCl, menadione and UV stress. Increased cell survival and decreased chlorophyll degradation in transformants under acclimated conditions further confirmed the relevance of *codA* in imparting stress tolerance. Our results indicated that the relevance of stress responsive genes can be efficiently validated for diverse abiotic stresses using *Chlamydomonas* system.

Keywords *Chlamydomonas* · Choline oxidase gene *codA* · Functional-genomics · Stress-genes · Transgenic-testing

Abbreviations

APX	Ascorbate peroxidase
codA	Choline oxidase A
HSP	Heat shock protein
LEA	Late embryogenesis abundant
MDA	Malondialdehyde
ROS	Reactive oxygen species
SSC	Saline sodium citrate
TAP	Tris–acetate–phosphate
SOD	Superoxide dismutase

Introduction

Mechanisms controlling plant abiotic stress tolerance are multigenic (Wang et al. 2003). With advances in genomics, several stress responsive genes have been identified and many stress databases provide clues about the nature of stress genes. About 100,000 expressed sequence tags responding to abiotic stresses were isolated from model plants (Wong et al. 2005). The stress transcriptome is diverse and many functionally different genes are expressed

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because the mechanisms of adaptation are many (reviewed in Bartels and Sunkar 2005; Vinocur and Altman 2005). Annotated information from diverse stress libraries provide leads about the nature of expressed genes and their putative function, but their relevance and functionality under stress is not understood. Although the functional significance of a gene in stress tolerance can be elucidated using mutants and other loss of function studies, overexpression studies can provide additional information about whether the gene expressed is relevant for imparting higher level of tolerance.

Availability of efficient gene transfer protocols in tobacco and high throughput in planta transformation in *Arabidopsis* facilitated the assessment of the relevance of diverse stress responsive genes by overexpression studies. Yeast and the moss model (*Physcomitrella patens*) have been used for functional testing of abiotic stress-related genes. Availability of diverse yeast mutants for complementation studies has provided large-scale options for assessing the relevance of genes using this system. In addition *Synechococcus* and *Chlamydomonas reinhardtii* could be other potential systems relevant for functional genomics. The unicellular green alga *Chlamydomonas* has been a useful model system to study many eukaryotic processes at molecular level (Harris 1989; Gutman and Niyogi 2004). It is being used as an experimental organism for studying the genetics and molecular biology of a number of cellular processes, like photosynthesis and chloroplast development, flagellar assembly and motility (reviewed in Grossman 2005). *Chlamydomonas* has been shown to be a suitable system for overexpression studies (Siripornadulsil et al. 2002; Kumar et al. 2005) or down regulation studies (Schroda et al. 2002; Shrager et al. 2003).

Chlamydomonas grows rapidly both photoautotrophically or heterotrophically and is amenable to classical genetic analysis. It is haploid during vegetative growth and allows any mutation to be immediately detected. Antisense or RNAi approaches were used to study the gene function (Schroda et al. 2002), and both forward and reverse genetic analyses were demonstrated (Pazour and Witman 2000) in *Chlamydomonas*. Although silencing of foreign genes is common, mechanisms of epigenetic gene silencing in *Chlamydomonas* are being probed (Scharf et al. 2000). Schroda et al. (2002) addressed this issue by developing HSP70 promoter fused with upstream region of other promoters. The *Chlamydomonas* genome project reported the complete sequence of chloroplast genome (Maul et al. 2002) and recently the whole genome (Grossman 2005; Joint Genome Institute, http://www.jgi.doe.gov/chlamy). Genomic sequence information and mutants are available at the *Chlamydomonas* stock center (Harris 1989, 2001). The expressed sequence tags (ESTs; Shrager et al. 2003), microarray information and other genomic resources can be found at http://www.chlamy.org and http://genome.jgi-psf.org/chlrez.infor.html.

Apart from this, *Chlamydomonas* could be a suitable organism to efficiently study stress effects. The response of *Chlamydomonas* organellar genomes to abiotic stimuli was demonstrated at transcriptional and post transcriptional levels (Lilly et al. 2002). Several methods often used to quantify the stress responses like chlorophyll content, estimation of malondialdehyde (MDA), osmolytes (Siripornadulsil et al. 2002) and phenotypic observation (Prieto et al. 1996) have been effectively employed even in *Chlamydomonas*. Besides growth measurements and counting the number of colonies regenerated on exposure to stress, other empirical assessment tools provide option for rapid screening of the transformed cell lines. Stress adaptation at cellular level can be studied using *Chlamydomonas* like any other plant system. Being a photoautotropic system, it has added advantages over yeast to understand the functional relevance of stress genes. Photoautotrophs, when exposed to abiotic stress (high light), generate reactive oxygen species (ROS), the primary cytotoxic compounds of oxidative stress. Because the chloroplast-mediated process generates high levels of ROS under stress, photoautotrophs are the desired systems to assess the relevance of plant stress responsive genes. To demonstrate this we use a well-characterized stress responsive gene, choline oxidase A (*codA*) in this study. We analyze the tolerance of *codA* expressing *Chlamydomonas* transgenic cells to various stresses under acclimation conditions in order to provide experimental evidence for recent stress concepts and to validate *Chlamydomonas* as a potential model system for the study of stress-responsive genes under different abiotic stresses. Stress effects can accurately be studied in these acclimated cells by prior exposure of the cells to sub-lethal stress levels.

Materials and methods

Chlamydomonas growth and culture conditions

The *Chlamydomonas reinhardtii* wild type (WT) strain CC-124 (mt^-) was used for all the experiments (*Chlamydomonas* center, Duke University, Durham, NC, USA). The cultures were photoautotrophically grown in liquid Tris–acetate–phosphate (TAP) medium (Harris 1989) in an incubator shaker with 80 rpm at 23°C under continuous illumination using cool fluorescent light with 40 W bulbs having light intensity of 100 μE m^{-2} s^{-1} and solid cultures were maintained on TAP–agar medium with the same light and temperature conditions. The culture medium did not contain sucrose or glucose and the cells were grown completely autotrophically throughout the experiment. Cultures

for the experiments were initiated from a single colony. Cells between the mid-log and late-log phases of growth with a known density were used for the experiments.

Plasmid constructs

Binary vectors pCAMBIA1301 harboring *gus* gene (Lin and Zhang 2005; kindly provided by Dr. M.V. Rajam, DU, New Delhi) and pGAH harboring *codA* gene (Hayashi et al. 1997; kindly provided by Professor Norio Murata, National Institute for Basic Biology, Japan) with hygromycin selectable marker driven by CaMV 35S promoter and NOS terminator were used for *Agrobacterium*-mediated transformation. The *codA* (choline oxidase gene) is from *Arthrobacter globiformis* (NCBI Accession # X84895). The pGAH/*codA* has a sequence encoding the transit peptide of the small subunit of RuBisCO. These vectors were mobilized into *Agrobacterium* strain EHA101 for further co-cultivation with *Chlamydomonas*.

Hygromycin sensitivity test: 15-day-old *Chlamydomonas* liquid culture was centrifuged and an equal amount of culture (having $\sim 4 \times 10^4$ cells) were plated on TAP solid medium having different concentrations of hygromycin (Sigma, St Louis, MO, USA). At the end of the selection period, the survival of colonies was recorded. Based on this, optimum concentration of hygromycin for selection of transformed colonies was determined.

Genetic transformation

Nuclear transformations were done by co-cultivating *Chlamydomonas* with *Agrobacterium* as described in Kumar et al. (2004, 2005). In brief, $\sim 10^7$ *Chlamydomonas* cells were plated on the solid TAP medium with 100 μM acetosyringone (Sigma) and were incubated under normal growth conditions for 5 days to allow a lawn of cells to be formed. An overnight grown *Agrobacterium* culture (OD_{600} 0.6) in AB minimal medium (Sambrook and Russel 2001) was spun down and resuspended in liquid TAP medium. The bacterial suspension (200 μl) was plated on the layer of *Chlamydomonas* growing on agar plates and co-cultivated for 48 h. At the end of co-cultivation, cells were washed in liquid TAP medium containing 500 μg ml^{-1} cefotaxime and centrifuged at 3,300*g* for 5 min; the pellet was resuspended in liquid TAP medium and was plated on solid TAP plates containing 10 μg ml^{-1} hygromycin and 500 μg ml^{-1} cefotaxime. Transformed colonies appeared on selection medium at the end of 7 days. Pure transgenic algal cells were completely recovered after transformation and only axenic cultures of algal transformants were used for experiments. Adequate care was taken to avoid artifacts due to bacterial contamination in the transgenic algal lines during subsequent experiments. Also, several tests were performed to make sure that the transgenic algal cultures were free from contaminants (see "Results").

Analysis of T-DNA integration and transgene expression

Genomic DNA isolation

Chlamydomonas cells were harvested by centrifugation and resuspended in Tris EDTA NaCl (TEN) buffer (10 mM Tris–HCl, 10 mM EDTA and 150 mM NaCl) and spun down immediately. Further the pellet was suspended in SDS-EB buffer (2% SDS, 400 mM NaCl, 40 mM EDTA, 100 mM Tris–HCl) and treated with chloroform:isoamyl alcohol. Further DNA was precipitated with 100% ethanol and dissolved in Tris EDTA (Harris 1989).

PCR analysis

To confirm integration of the *gus* gene in transformed *Chlamydomonas* colonies, PCR analysis was performed with primer pairs 5′-GGG CAG GCC AGC GTA TCG TG-3′ and 5′-GTC CCG CTA GTG CCT TGT CCA GTT-3′ to amplify 680 bp fragment of the *uidA* (referred here as *gus*) gene. To confirm integration of *codA* in transformants, the primer pairs 5′-TCG ACA TCG AGA ACC TG-3′ and 5′-TGG AGA GCA CGA CTT CAT TG-3′ were used to amplify 780 bp fragment of the *codA* gene. Further, to confirm equal loading of DNA and to know the targeted nuclear transformation, the primer pairs 5′-CTG GAG AAG ACC TAC GAG C-3′ and 5′-GCA GGC CCT CGT TC ATC GAT-3′ were used to amplify 900 bp fragment of nuclear actin gene. The presence or absence of the *VirG* gene was confirmed by PCR using *Agrobacterium VirG* gene-specific primers as described in Siritunga and Sayre (2003).

Genomic DNA dot blot

Undigested genomic DNA (50 ng) was spotted on the nitrocellulose membrane (Hybond, Amersham) using dot blot apparatus and fixed by exposing to UV-radiation in an UV cross linker. Pre-hybridization was done at 42°C for 2 h and hybridization was done at 55°C over night using blocking solution (0.5 M Na phosphate buffer, 1 mM EDTA and 7% SDS) having pH 7.2. Probes (*gus* and *codA*) were prepared by PCR or random labeling of respective gene fragments with α-^{32}P-dCTP. The hybridized blot was washed in 2× saline sodium citrate (SSC) containing 0.1% SDS (w/v) twice for 10 min at 37°C. The blot was further washed sequentially with 2× SSC for 10 min at 37°C, 6× SSC for 10 min at 37°C; 4× SSC for 10 min at 37°C; 2× SSC for 15 min at 55°C; 0.2× SSC for 10 min at 55°C. Hybridization signals were detected following exposure to Kodak

X-ray film at −70°C for 18 h and developed by autoradiography (modified from Pazour and Witman 2000).

Southern blot

Genomic DNA (20 μg) of *codA* gene transformed and WT *Chlamydomonas* pure culture was digested with *Hin*dIII, electrophoresed on a 0.8% agarose gel and blotted on nitrocellulose membrane. The blots were pre-hybridized as previously described (in "Genomic DNA dot blot") and hybridization was done at 60°C for 16 h. Probe was prepared by radio labeling the *codA* fragment with α-^{32}P-dCTP by PCR. The hybridized blots were washed as mentioned earlier. The membrane was then exposed to Kodak X-ray film and developed by autoradiography.

Real time PCR

Total RNA from selected *codA* gene transformed lines and a WT *Chlamydomonas* was isolated using TRIZOL (Gibco BRL kit) according to manufacturer's instructions. First strand cDNA was synthesized from total RNA (200 ng) by oligo (dT) primers using Molony Murine Leukaemia Virus reverse transcriptase (MMLV-RT; MBI Fermentas, Hanover, MD, USA). The cDNA pool was used as a template to perform real time PCR (RT-PCR; Opticon2, MJ research, Watertown, MA, USA) and the log linear phase was set at 30 cycles. Real-time PCR was performed in the presence of the fluorescent dye SYBR-green (DyNAmo SYBR-green qPCR Kit, Finnzymes, Espoo, Finland) following manufacturer's protocol. Primer pairs 5′-TGA ACA CCC TGC GGC ACG GCT ACC-3′ and 5′-GGC GGG CTG GGC GGC GAT TTC-3′ that amplify 220 bp were used to quantify *codA* transcripts and the primer pairs 5′-GAG CCC GGC GCG ACA CCA C-3′ and 5′-CAC ATA TGC CTC ACC CAA ACA ACA-3′ that amplify 300 bp were used to quantify the Rubisco small sub unit (*rbcs*) transcripts and this was used as internal control. All reactions were heated to 95°C for 5 min followed by 30 cycles of 95°C for 1 min 30 s, 65°C for 1 min 30 s and 72°C for 1 min, final extension for 72°C for 10 min. The Ct value (the fractional cycle number at which fluorescence passes a fixed threshold) was used to compare the target RNA in the sample. The calculations were performed as described in the manufacturer's protocol book.

Estimation of glycinebetaine

Fresh *Chlamydomonas* cells were ground with 2 N H_2SO_4 and centrifuged (13,000*g*). To this supernatant, cold KI–I_2 reagent (15% iodine + 20% KI in water) was added and stirred in a vortex mixer and centrifuged (13,000*g* for 30 min). To the pellet, 1 ml of 1, 2-dichloroethane was added and vigorously vortexed frequently. After 2.5 h of incubation at room temperature the absorbance of this solution was measured at 365 nm using UV–visible spectrophotometer (UV2450, Shimadzu corporation, Kyoto, Japan). Reference standards of pure glycinebetaine (50–200 μg ml^{-1}) were prepared in 1 N H_2SO_4. To analyze the stability and reproducibility of absorbance values, we tested standard glycinebetaine solution (90 μg ml^{-1}) at various acid concentrations. Standard curves were used to quantify the glycinebetaine content in the *Chlamydomonas* samples. This method was modified from Grieve and Grattan (1983).

Stress experiments

For experiments, the *Chlamydomonas* cells just at the beginning of the stationary phase were used and their viability assured (data not shown). The cells were grown in TAP medium until the density of cells reached an OD_{750} of 1.5 which is equal to 50 ml of the culture with ~4 × 10^5 cells ml^{-1}. The cells were spun down and redissolved in 1 ml TAP medium. From this 40 μl of this culture (containing ~16,000 cells) was used for spotting and 100 μl was used for inoculating 50 ml liquid TAP medium. Culture (40 μl) spotted (1 cm radius) on a solid TAP medium gave adequate colonies to qualitatively assess the growth. The diverse stress effects were assessed either by estimating growth of the colonies on solid medium or by assessing growth pattern by quantifying the density of culture (OD_{750}) in a liquid medium. The protocol for cell growth measurement detailed in Siripornadulsil et al. (2002) was modified suitably and used in this study.

Stress imposition

The response of *Chlamydomonas* cells to different stresses namely PEG, NaCl, LiCl, menadione and methylviologen was studied after pretreatment with corresponding induction (acclimation) stress. After acclimation at corresponding mild stress levels, cells were spun down and subsequently exposed to severe stress levels for a definite period.

PEG stress

Osmotic stress response using PEG-8000 was studied at wide range of water potential (Ψw; −0.1 to −0.6 MPa). Prior to challenge with higher stress levels, the cells were acclimated at lower induction stress levels. The induction stress (MPa) levels were −0.05, −0.1 and −0.2 (for 8 h) and corresponding challenging stress (MPa) levels were −0.1 or −0.2, −0.3 or −0.4 and −0.5 or −0.6, respectively. The liquid simulated stress medium was prepared by

adding appropriate freshly prepared PEG stock solution into the autoclaved TAP medium. The final water potential (MPa) of the medium was measured using dewpoint WP4 potentia meter (Decagon devices, Washington DC, USA). PEG (semi) solid medium was prepared by adding appropriate PEG stock solution into the sterilized TAP medium containing phytagel (Hi-media laboratories Ltd, Mumbai, India).

NaCl stress

For salinity stress with NaCl the induction stress concentrations were 25, 50 and 100 mM for 8 h and corresponding challenging stress levels were 50, 100 and 150–300 mM, respectively.

LiCl stress

For LiCl-mediated ionic stress the induction stress levels were 5, 10 and 20 mM (for 8 h) and corresponding challenging stress levels were 10, 20 and 30 or 40 or 50 mM, respectively.

Menadione stress

The induction stress levels were 3, 5 and 10 μM for 8 h and challenging stresses levels were 5, 10 and 15 or 20 or 25 μM, respectively.

Methylviologen and high light stress

Chlamydomonas cells were exposed to an induction stress of 50, 100, 150 and 200 nM of methylviologen at a light intensity of 100 μE m^{-2} s^{-1} for 8 h and subsequently challenged with 200, 400, 600 and 800 or 1,000 nM of methylviologen with relatively higher light intensity of 300 μE m^{-2} s^{-1} respectively.

For PEG, NaCl, LiCl, menadione and high light stresses, the cells were continuously exposed to the stress treatment in liquid or solid TAP medium for definite periods until the observations were made.

Temperature stress

To study the cold stress response, initially the *Chlamydomonas* cells were exposed to cyclic induction temperature treatment [20°C (2 h) + 15°C (2 h) + 10°C (1 h)] and subsequently exposed to challenging stress at 7 or 5 or 3 or 0°C for 15 h. Similarly for high temperature stress, the *Chlamydomonas* cells were initially exposed to gradual induction temperature from 25 to 35°C over 3 h and subsequently exposed to 40°C for 1 or 2 h and at 42°C for 1 or 2 h.

UV stress

Initially the cells were exposed to low UV B (285 nm) irradiance 250 J (Herolab UV chamber, Wiesloch, Germany) and subsequently challenged with UV irradiance of 2,000 or 4,000 or 6,000 or 8,000 J and allowed to recover for defined period under normal light. On exposure to challenging UV stress treatment one sub set of the culture was inoculated in fresh liquid TAP medium to assess the growth pattern and another sub set was spotted on solid TAP for the phenotypic assessment of growth.

To study the response of high temperature, cold and UV stress, the cells were exposed to definite stress levels, transferred to liquid or solid TAP medium and allowed to recover under normal growth conditions. At the end of the definite recovery period, the growth response of cells in liquid and solid TAP medium was assessed.

Quantification of stress effects

Cell growth and chlorophyll content

Growth pattern was determined by measuring the absorbance at 750 nm (also described in Siripornadulsil et al. 2002). Chlorophyll was extracted from the known amount of cells in acetone:DMSO mix (1:1, v/v), centrifuged and the supernatant was used for estimation. The absorbance was recorded at 663, 645 and 553 nm using UV–visible spectrophotometer and total chlorophyll was estimated and expressed in mg g^{-1} fresh weight (modified from the protocol used by Yoshimura et al. 2004).

Cell viability

Cell viability was measured by 2,-3,-5-triphenyltetrazolium chloride (TTC) (Sigma, Aldrich, India). TTC assay solution (0.1%) was prepared by dissolving TTC in sodium phosphate buffer (pH 7.4). *Chlamydomonas* cells exposed to NaCl and high temperature stress were initially washed in sterile water and incubated in the TTC solution at room temperature for 5 h in dark. Subsequently these cells were washed to remove unbound TTC and boiled with 5 ml of 2-methoxy ethanol until dryness to extract bound formazon. Another 5 ml of 2-methoxy ethanol was added and absorbance was measured at 485 nm using UV–visible spectrophotometer (Senthil-Kumar and Udayakumar 2004).

Superoxide radical quantification

To quantify the superoxide radical levels in the *Chlamydomonas* cells, XTT sodium 3′[1-(phenylamine-carbonyl-3, 4-tetrazolium)-bis [4 methoxy-6-nitro] benzene sulfonic acid hydrate] assay (cell proliferation kit II, Roche

Diagnostics Corporation, Basel, Switzerland) was done (modified from Schopfer et al. 2001). The WT and *codA* transformed cells were incubated in 1 ml of medium containing 300 nM methylviologen, 100 μM XTT and 25 μM PMS (*N*-methyl dibenzopyrazine methyl sulfate) in 20 mM potassium phosphate buffer pH 6.0 and exposed to high light stress (700 μE m^{-2} s^{-1}) for 6 h. Further the extent of reduction of XTT to formazon giving pink color was quantified using a micro titer plate reader (Sunrise-Magellan, Tecan, Australia) at 470 nm (protocol also described in Senthil-Kumar and Udayakumar 2006).

Membrane integrity

The membrane damage of cells due to stress was assessed using non-toxic water-soluble dye Evans blue, which diffuses through the damaged membrane and accumulates as blue protoplasmic stain. The method previously described (Senthil-Kumar et al. 2003) was modified to assess membrane damage of *Chlamydomonas* cells exposed to cold stress. The protocol involves incubating the stressed cells in 0.05% of Evans blue for 2 h and the dye bound to damaged cells was solubilized by incubating in 100% methanol for 30 min at 50°C. The dye content was quantified by measuring absorbance at 600 nm using UV–visible spectrophotometer.

Results

Chlamydomonas growth

The cells from a single colony were photoautotrophically grown in liquid medium for 15 days and subsequently serial dilutions were made and plated. The number of colonies developed after 7 days were recorded to arrive at the growth. The results showed that an aliquot of 1 ml of such cultures had $\sim 4 \times 10^6$ cells. For the phenotypic growth observations, corresponding treatments were also simultaneously maintained in liquid medium and growth rates were quantified by daily growth measurements.

Stress responses of *Chlamydomonas*

In case of PEG, NaCl, LiCl, menadione and methylviologen, induced stress treatments, the growth response was examined at the end the of stress period, while the recovery growth after exposure to challenging stress was examined for heat, cold and UV stress. In liquid cultures, daily measurements were made either during stress or during recovery period. Growth of *Chlamydomonas* was substantially decreased in all the stresses. However the extent of growth reduction varied in different stresses (Fig. 1).

Osmotic stress: PEG

WT cells of *Chlamydomonas* were induced (acclimated) at lower levels of simulated osmotic stress with PEG and subsequently challenged with severe stress (low Ψw). Gradual reduction in algal growth rate was seen as the stress levels increased (Figs. 1a, 2a). Reduction of growth was observed even at relatively low levels of stress ($\Psi w = -0.2$ MPa). Although the growth inhibition was high during initial stages ($\Psi w = -0.4$ MPa), the cells recovered and recorded relatively higher growth. However, high levels of osmotic stress ($\Psi w = -0.6$ MPa) led to severe reduction in growth (Fig. 2a) and chlorophyll degradation (data not shown).

Salinity stress (osmotic and ionic): NaCl

The phenotypic growth observations showed severe growth reduction even at 150 mM NaCl and at the subsequent severe stress levels, the growth was completely arrested (Fig. 1a). The algal cells grown under relatively lower stress levels (100 mM) had shown less reduction in growth and the response was gradual. There was a decrease in the developmental rate of colonies and the growth rate of cells was significantly less at the stationary growth phase. The reduction in growth was high at severe stress levels (200 mM) (Fig. 2a).

LiCl (ionic effect)

Phenotypic observations clearly indicated that at 10 mM LiCl, the growth reduction was only marginal and at 20 mM and beyond, drastic reduction in growth was observed (Fig. 1a). This was also clearly reflected in the cell growth pattern (Fig. 2a).

Oxidative stress: menadione

Menadione, a quinone compound gets reduced to hydroquinone or semiquinone by cellular reductases deriving electrons from NADPH. Both hydroquinones and semiquinone radicals undergo auto-oxidation with the regeneration of parent quinone and formation of superoxide radicals and hydrogen peroxide (Shi et al. 1994). The oxidative stress effects of menadione were studied with prior induction treatments. Both phenotypic growth observations and cell growth pattern measurements indicated that lower concentrations of menadione (5 μM or below) had a marginal effect on the algal growth, but concentrations beyond 10 μM substantially reduced the growth (Figs. 1a, 2a).

Oxidative stress: methylviologen with high light

The algal cells showed drastic reduction in the growth during stress even at low levels of methylviologen (200 nM)

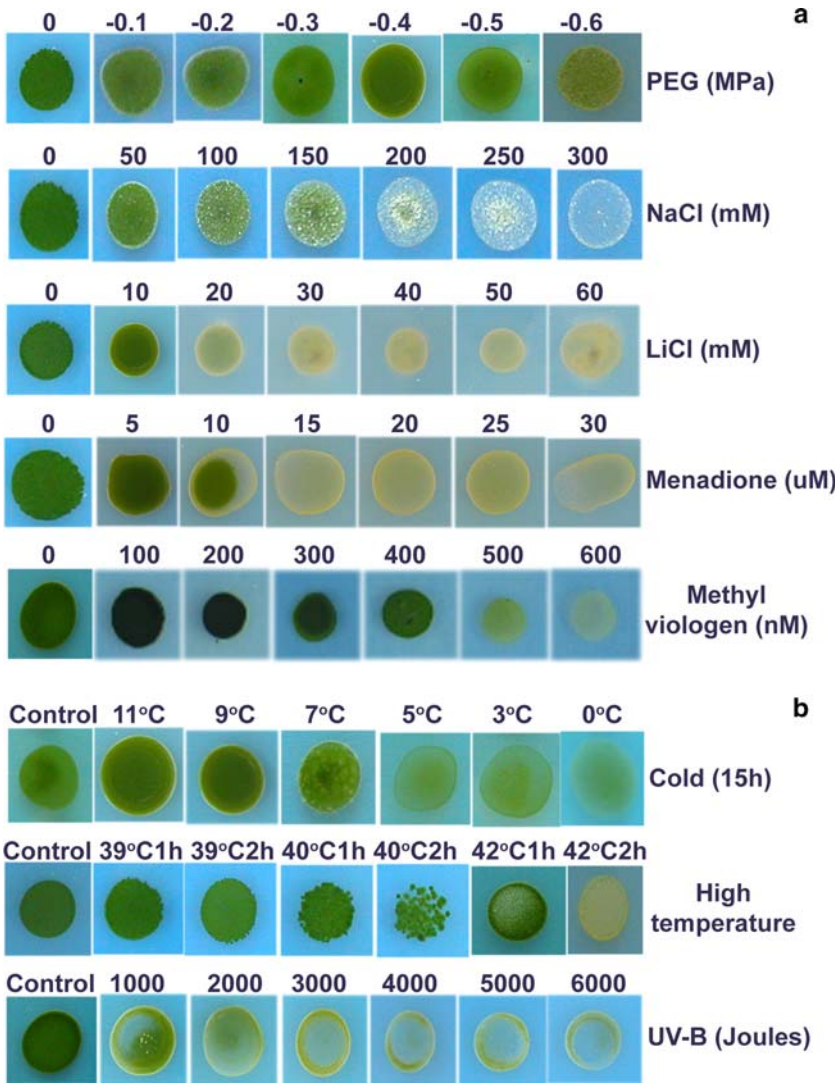


Fig. 1 Phenotypic response of WT *Chlamydomonas* on exposure to different abiotic stresses. *Chlamydomonas* CC124 cells exposed to prior induction (acclimation) stress were spotted on the TAP medium with different concentrations of PEG, NaCl, LiCl, menadione and methylviologen and stress responses was studied for 7 days. The methylviologen treated cells were maintained under relatively high light (300 μE m^{-2} s^{-1}). At the end of stress period the photographs were taken (**a**). Cells acclimatized to the respective induction stress were exposed to the indicated stress levels of cold or high temperature or UV-B and spotted on the TAP medium. The cells were allowed to recover for 3 days (cold or high temperature) and for 5 days (UV-B). At the end of recovery period the photographs were taken (**b**)

with high light. The growth attained a plateau at higher stress levels (>400 nM) and only a narrow and short log phase of growth was seen at these stress levels (Figs. 1a, 2a).

Cold stress

Although initially there was a steady increase in growth, it reached a plateau with early onset of slow growth. The recovery growth was negligible in cells exposed to severe stress (0°C) levels (Figs. 1b, 2b).

High temperature stress

At all the stress levels the recovery growth on exposure to high temperature following an optimum induction temperature treatment showed a similar trend. However, stress levels beyond 41°C (1 h) caused more than 50% growth reduction and reached a plateau at stationary growth phase with OD_{750} of 0.5 (Fig. 2b). At higher stress levels [42°C (1 h) and 42°C (2 h)] the recovery growth was almost negligible (Fig. 1b). The non-induced algal cells directly exposed to severe stress treatment had much less recovery growth and also more chlorophyll degradation compared to the induced cells. Also, the induced cell culture reached the stationary phase earlier, whilst the non-induced took much longer time to reach the same growth status (data not shown). Clear acclimation response was seen in the pre-induced cells and the growth reduction was much larger in the non-acclimated cells (Supplementary Fig. 5).

UV stress

Growth inhibition was noticed even at 1,000 J in spite of prior induction with lower UV levels. In all the stress levels studied, the growth of cells reached early stationary growth phase after 4 days of inoculation and reached a plateau. At higher irradiance of UV 6,000 J, growth was completely arrested (Figs. 1b, 2b).

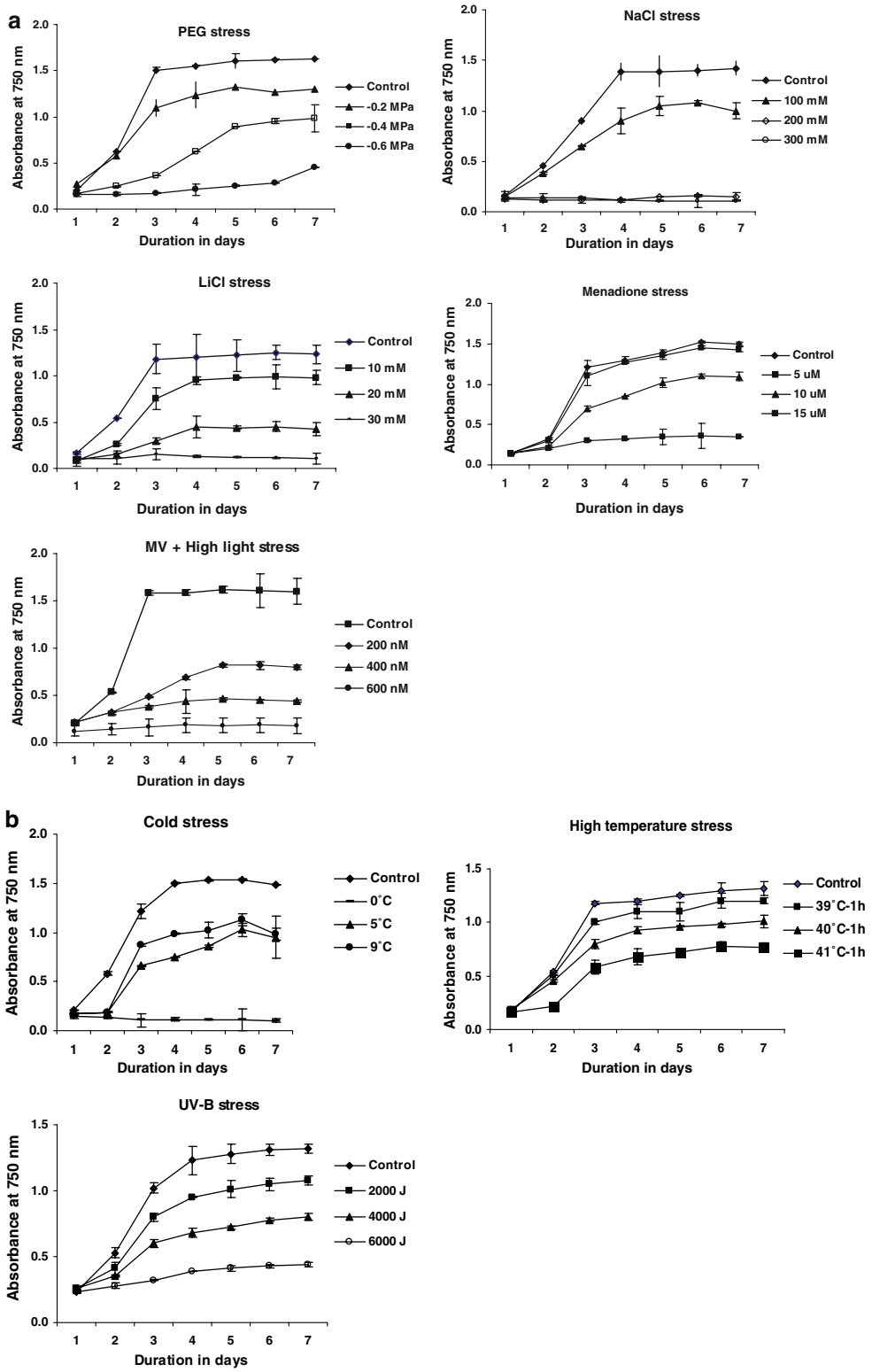


Fig. 2 Growth response of WT *Chlamydomonas* to different abiotic stresses. *Chlamydomonas* cells exposed to respective prior induction stresses were inoculated into the liquid TAP medium with PEG or NaCl or LiCl or menadione or methylviologen (*MV*) and allowed to grow for 7 days. The growth measurements during stress were made on daily basis (**a**). The prior-induced cells were imposed with cold or high temperature or UV-B stress and allowed to recover in the liquid medium. Growth during recovery was measured by taking absorbance at 750 nm on daily basis (**b**). The stress protocols were same as described in Fig. 1. The values are from two independent experiments and the *bars* represent standard error

Based on the response of *Chlamydomonas*, the optimum challenging stress levels for different stresses were determined. More than 50% reduction in growth was seen at PEG-0.6 MPa, 150 mM NaCl, 20 mM LiCl, 15 μM menadione, 400 nM methylviologen at 300 μE m^{-2} s^{-1}, heat stress for 40°C (2 h), cold stress of 5°C (15 h) and 4,000 J of UV.

External supply of glycinebetaine imparts salinity tolerance in *Chlamydomonas*

To study the role of glycinebetaine in stress tolerance, the growth medium with NaCl was supplemented with 500 or 1,000 mg l^{-1} glycinebetaine. The *Chlamydomonas* cells grown on the medium supplemented only with glycinebetaine (500 mg l^{-1}) showed normal growth; however, at higher concentrations (1,000 mg l^{-1}), marginal growth reduction was noticed. Although NaCl substantially reduced the growth, with glycinebetaine significant increase in growth was observed (Fig. 3a, b). The results substantiate that glycinebetaine may impart salinity tolerance in *Chlamydomonas*. However, the cells enriched with glycinebetaine did not show improved growth when subjected to LiCl and menadione (data not shown).

Genetic transformation of *Chlamydomonas* with *gus* gene

Sensitivity of Chlamydomonas to hygromycin

Chlamydomonas cells showed declined growth at 5 μg ml^{-1} of hygromycin and cell growth was completely inhibited at 15 μg ml^{-1} (Supplementary Fig. 1). Cell growth measurements in liquid cultures clearly indicated the gradual reduction in multiplication rate at 5 and 7 μg ml^{-1} hygromycin and complete arrest of growth at 10 μg ml^{-1} and beyond. Subsequent selection of the transformants was carried out at 10 μg ml^{-1} hygromycin.

Transformants expressing gus gene

PCR analysis was performed to screen a large number of lines and only two representative lines were selected for further experiments. These selected lines when reassessed, survived on the antibiotic supplemented medium and growth of transformants in liquid medium with hygromycin reached early stationary growth phase (with OD_{750} = 1.5) in 5 days. Amplification of 680 bp fragment by PCR in both the transformed lines (*gus* 1–7 and *gus* 1–10) confirmed the genetic transformation. Further dot blot analysis of genomic DNA from the transformed cells with radiolabelled *gus*

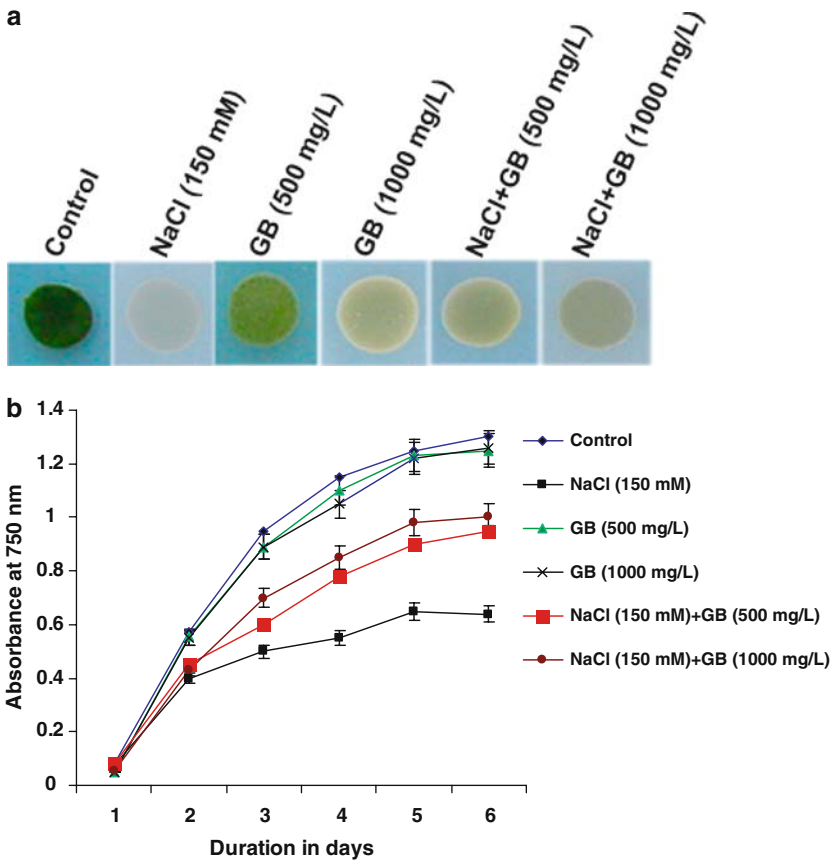


Fig. 3 Effect of glycinebetaine on NaCl stress response of *Chlamydomonas*. *Chlamydomonas* WT cells were pre-induced with lower concentrations of NaCl and subsequently spotted on TAP medium containing 150 mM NaCl with or without glycinebetaine. Growth was recorded at the end of 4 days of stress (**a**). Another set of pre-incubated cells were inoculated into TAP liquid medium containing NaCl with or without glycinebetaine and daily growth rate was measured (**b**). The values are representative of two independent experiments and the *bars* represent standard error

gene probe showed clear hybridization signals, there by substantiating efficient transformation of *gus* gene in the genome (Supplementary Fig. 2a–c). The relative efficiency of *Agrobacterium*-mediated transformation ranged from 7 to 8 cells per 10^6 cells.

Chlamydomonas transformants expressing *codA*

Molecular analysis of codA expressing transformants

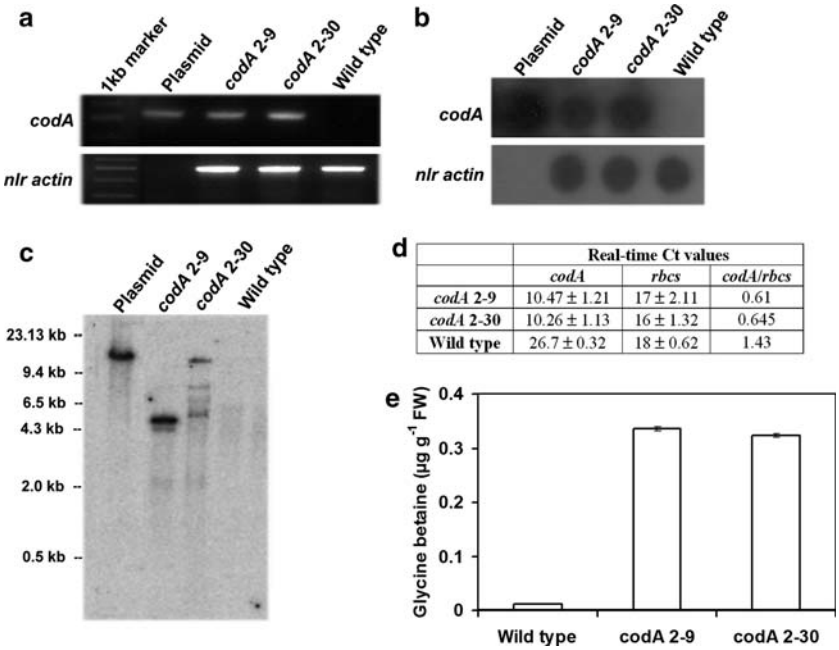
PCR analysis of the selected *codA* transformed lines using specific primers showed the presence of *codA* gene fragment (Fig. 4a). Dot-blot analysis using *codA* specific probes also showed hybridization signals in the transgenic lines (Fig. 4b). Southern analysis using *Hin*dIII digested DNA of transgenic line *codA* 2–9 showed single integration and *codA* 2–30 had two copies in the genome (Fig. 4c). Quantitative real-time PCR analysis was performed to assess the extent of *codA* transcript expression in the transformed *Chlamydomonas* cells. The real-time Ct values of *codA* transformed lines showed early onset of cDNA amplification, which indicated higher expression levels of *codA* transcripts (Ct = 10). There was no amplification of *codA* transcripts (Ct = 26) in the WT (Fig. 4d). Two positive colonies multiplied on hygromycin medium (lines *codA* 2–9 and *codA* 2–30) were used for further experiments.

Screening for bacterial contamination

Cefotaxime concentrations adequate enough to completely arrest the bacterial growth were used to select single pure *Chlamydomonas* colony and axenic cultures were obtained. To ensure that the transgenic algal cultures were not contaminated with *Agrobacterium*, several tests were performed. Transformed culture streaked on nutrient rich LB medium did not show any bacterial growth even after 4 days of incubation. The absence of *Agrobacterium* contamination was also verified by *Agrobacterium VirG*-specific primers (Supplementary Fig. 3a, b). PCR amplification, genomic dot blot and Southern analysis to identify possible fragment outside the T-DNA did not show any signals (data not shown). This ruled out the possibility of *Agrobacterium* contamination in the selected transgenic culture. Moreover it is unlikely that the constructs (with intron) used in this study were expressed to produce functional protein in prokaryotic bacteria. The observed results demonstrated the integration of T-DNA in transgenic cultures and ruled out bacterial contamination (Supplementary Fig. 3a, b).

Glycinebetaine

Glycinebetaine levels were measured in two lines expressing *codA* gene. Transformed lines showed higher level of



	Real-time Ct values		
	codA	*rbcs*	*codA/rbcs*
codA 2-9	10.47 ± 1.21	17 ± 2.11	0.61
codA 2-30	10.26 ± 1.13	16 ± 1.32	0.645
Wild type	26.7 ± 0.32	18 ± 0.62	1.43

Fig. 4 Molecular analysis of *Chlamydomonas* transformants expressing *codA* gene. Two *codA* transgenic lines were selected to study the nuclear integration and expression. PCR amplification using *codA* specific primers (**a**) and dot-blot analysis (**b**) were performed to confirm the genetic transformation. Transgene integration was also tested by Southern analysis (**c**) and transcript expression pattern was studied using real time PCR. The Ct values of *codA* gene and *rbcs* (internal control) were determined simultaneously from the common cDNA pool. Each value represents the mean standard deviation values of three independent experiments (**d**). The glycinebetaine levels were estimated by colorimetric method; the *error bar* represents the standard deviation values (**e**). *rbcs* Rubisco small subunit

accumulation of glycinebetaine, whereas only negligible levels were noticed in WT (Fig. 4e). Higher levels of glycinebetaine in transgenic algae did not impair growth of the cells under non-stress conditions.

Response of *codA* 2–9 transgenic line to different abiotic stresses

Salinity stress

The phenotypic observations clearly indicated that the *codA* transformed cells showed better growth at 150 mM NaCl compared to WT. The growth rate of transgenic algae was much higher and the cells reached OD_{750} of 0.8 in 4 days, whilst the WT took 6 days to reach the same level of growth. Also, the transformed cells retained higher chlorophyll content at the end of stress (150 mM). Further these cell lines maintained higher cell viability even under severe stress levels. But, at high NaCl levels (200 mM) the transgenic cells showed poor growth and high cell mortality (Table 1), however the surviving cells retained higher total chlorophyll although the growth was arrested (Table 1).

Methylviologen and high light stress

Oxidative stress response of *codA* expressing *Chlamydomonas* transformants was studied by exposing to high light with methylviologen. Phenotypic observations showed that at lower concentrations of methylviologen (300 nM) there was not much difference in the performance of the WT and transgenic algae. However, at higher levels of methylviologen (400 nM) transgenics showed higher growth as compared to WT (Supplementary Fig. 4a). The extent of chlorophyll degradation was also much less in transgenic lines as compared to WT (Table 1). Although differences in accumulation of superoxide radical levels between WT and transgenic were only marginal at 300 nM methylviologen, the transformants accumulated relatively less superoxide radicals at 400 nM methylviologen (Table 1). This indicated that *codA* gene expression in the transgenic algae provided adequate cellular level tolerance under methylviologen-induced oxidative stress.

Cold stress

There was no growth difference noticed between WT and transgenic algae (Supplementary Fig. 4b). However the growth pattern showed marginal increase in recovery response of the transgenic algae compared to WT (data not shown). The transgenic algae also retained higher chlorophyll content under stress. The Evans blue results also showed less membrane damage in transgenic algae (Table 1). From this study it can be inferred that, cold tolerance of transgenic cell lines was seen only upon induction

Table 1 Effect of NaCl, methylviologen (MV) with high light, high temperature (HT) and cold stress on transgenic *Chlamydomonas* expressing *codA* gene

	Wild type	*codA* transgenic	Wild type	*codA* transgenic
Salt stress	Chlorophyll (% reduction over control)		TTC assay (% reduction in A_{485} over control)	
NaCl (150 mM)	31.03 ± 2.4	18.18 ± 0.32	59.05 ± 1.05	28.57 ± 2.03
NaCl (200 mM)	56.89 ± 0.04	41.81 ± 0.21	78.07 ± 0.65	71.42 ± 1.20
MV with high light	Chlorophyll (% reduction over control)		XTT assay (fold increase in A_{470} over control)	
MV (300 nM)	44.06 ± 1.50	20.21 ± 1.20	2.97 ± 0.30	2.58 ± 1.20
MV (400 nM)	55.35 ± 0.60	34.21 ± 0.20	5.57 ± 0.45	3.21 ± 0.70
Cold stress	Chlorophyll (% reduction over control)		Evans blue test (fold increase in A_{600} over control)	
3°C (15 h)	49.50 ± 0.50	34.01 ± 0.20	1.67 ± 0.23	0.75 ± 0.01
5°C (15 h)	45.10 ± 0.45	30.60 ± 0.90	1.19 ± 0.63	0.40 ± 0.01
HT stress	Chlorophyll (% reduction over control)		TTC assay (% reduction in A_{485} over control)	
40°C (2 h)	53.24 ± 1.20	24.05 ± 0.54	53.24 ± 0.23	25.35 ± 0.3
42°C (1 h)	63.22 ± 0.45	35.78 ± 1.05	75.00 ± 0.47	63.07 ± 0.1

The transgenic *Chlamydomonas* expressing *codA* gene (*codA* 2–9) was inoculated into liquid TAP medium containing NaCl (150 or 200 mM). After 7 days the stress response was analyzed by estimating the chlorophyll content and cell viability by TTC assay. To study the oxidative stress response, cells were inoculated on methylviologen (300 or 400 nM) medium and exposed to relatively high light (300 μE m^{-2} s^{-1}). At the end of 7 days of stress, the chlorophyll content was estimated. For XTT assay the cells were initially induced with 100 nM methylviologen under 100 μE m^{-2} s^{-1} light intensity and subsequently challenged with high stress levels (300 or 400 nM methylviologen + 1,200 μE m^{-2} s^{-1} light intensity) for 3 h. At the end of stress the XTT reduction was quantified. The cold tolerance was studied by exposing the cells to cyclic induction temperature and challenging with temperature 3 or 5°C. Total chlorophyll and membrane damage by Evans blue assay were performed after 24 h of recovery. In the high temperature stress, the cells were spun down after the stress and inoculated to fresh TAP medium. At the end of 36 h of recovery, cell viability by TTC reduction and chlorophyll content were assessed. The values are mean of two independent experiments ± SD

A_{485} absorbance at 485 nm

and the differences were marginal between WT and transgenic lines under non-induced conditions.

High temperature stress

Our earlier observations showed that at 41°C the reduction in recovery growth of induced cells was high. Hence, thermotolerance of *codA* transformants was also studied at relatively higher temperature levels (42°C). Phenotypic observations indicated that there was marginal increase in the growth of transgenic cells compared to WT at 40°C (2 h). At higher stress levels [42°C (1 h)], the transformed cells showed reduced growth (Supplementary Fig. 4b). However, *codA* transformants showed less reduction in chlorophyll and higher cell viability at all the stress levels studied. At 42°C (1 h) the cell viability substantially decreased in transgenic algae. Interestingly under non-induced conditions both WT and transgenic algae showed similar growth rate, chlorophyll retention and cell viability (Table 1). Particularly, the performance of non-induced transgenic algae was on par with WT at high stress levels [42°C (1 h)] (data not shown). This clearly indicated that the thermotolerance of transformed cells should be assessed only upon acclimation stress.

There was no difference in the performance between transgenic algae expressing *codA* gene and WT under PEG-induced osmotic stress, LiCl-induced ionic stress, menadione-induced oxidative stress and UV-induced ionizing irradiation stress (data not shown).

Discussion

Among several approaches available to characterize the large number of stress responsive genes, up-regulation studies are one of the approaches and provide information whether the altered expression results in a resistant phenotype. However, many of the model plant systems used for such studies require elaborate protocols to obtain stable transformants and warrant analysis of large number of individual transgenics. Amongst the unicellular organisms amenable for high throughput screening, *Chlamydomonas* could be an ideal option and being a photoautotroph, the relevance of stress responsive genes can be well characterized.

Chlamydomonas is a suitable system to study the abiotic stress responses in vivo

The response of *Chlamydomonas* to several abiotic stresses like high and low temperature, osmotic stress; salinity and heavy metal toxicity has been examined in several studies (Prieto et al. 1996; Goho and Bell 2000; Siripornadulsil et al. 2002). However, in all these studies, cells were directly exposed to severe stress levels and growth response was studied. Many studies have shown the ability of plants and other organisms to tolerate otherwise lethal stress upon prior exposure to sub lethal stress often referred to as induction stress (reviewed in Bartels and Sunkar 2005; Senthil-Kumar et al. 2007). During induction stress, many stress-responsive genes are expressed which, in-turn trigger several physiological and biochemical processes relevant for stress tolerance. Such an acclimation response is ubiquitous. Genetic variability can be best studied only upon exposure to prior induction stress (Senthil-Kumar et al. 2003; Bartels and Sunkar 2005). The need to study the relevance of stress genes in the background of changes in stress transcriptome that occur during induction period has been increasingly realized recently.

Based on the concept of acclimation response, specific stress protocols were employed to assess the basal tolerance (under non-induced conditions) and acquired tolerance (upon induction). In this study, the induction response was clearly noticed in *Chlamydomonas* for all the stresses studied. Survival under stress and recovery response reflects both basal and acquired tolerance. The cells exposed to prior temperature induction treatment performed better than the subsequent severe stress and the non-induced cells showed higher stress damage (Supplementary Fig. 5). Acclimation response of *Chlamydomonas* photosynthetic machinery to high temperature has also been shown in a previous study by Tanaka et al. (2000). Besides we also demonstrate that acclimation response in *Chlamydomonas* in all the stresses studied. The acclimated cells showed higher growth at the challenging stress levels compared to non-acclimated cells.

In the present study, concentration dependent decrease in growth of *Chlamydomonas* was noticed when subjected to simulated osmotic stress-induced by PEG (Fig. 1a). The *Chlamydomonas* system has specific advantage to efficiently study the stress responses of cells induced by simulated osmotic stress by PEG treatment, as the whole cells can experience stress unlike higher plants where only roots are exposed to such stress and the effects observed in other plant parts are only indirect.

In the NaCl-supplemented medium, cells experience an initial osmotic stress followed rapidly by ionic stress. Because LiCl does not contribute to osmotic stress in the stress medium (Prieto et al. 1996) the cells experience only ionic stress. In this study NaCl stress led to reduced growth and less cell viability under stress compared to LiCl stressed cells, indicating that observed decrease in growth was largely an osmotic effect.

Almost all the environmental stresses lead to production of ROS and thereby oxidative stress (reviewed in Mittler 2002). Dissipation of excess energy absorbed by the

photosynthetic apparatus is essential for the survival of almost all photosynthetic organisms. It prevents photooxidative damage that occurs when excited chlorophyll molecules improperly transfer their higher energy state to oxygen and convert into ROS (Asada 1999). In photoautotrophic organisms, the major source of ROS is light reactions and also photorespiration (Mittler 2002; Rizhsky et al. 2003). In this regard *Chlamydomonas* is a suitable photoautotroph to study the effects of oxidative stress, which occurs when exposed to any abiotic stress under high light. Chloroplast-mediated ROS generation in photosynthesizing cells in excess light is accelerated by methylviologen as it diverts the electrons from photosynthetic electron transport chain to free oxygen and hence is widely used to assess the oxidative stress response in photosynthesizing tissues (Yoshimura et al. 2004). Significant reduction in growth of *Chlamydomonas* cells observed when exposed to methylviologen under moderate light intensity (Figs. 1a, 2a) suggests that it is a potential system to study the oxidative stress responses. Menadione, another compound widely used to generate superoxide radicals (Reichheld et al. 1999; Senthil-Kumar et al. 2003) in eukaryotic cell cultures, also reduced the growth, chlorophyll content and cell viability in *Chlamydomonas* (Figs. 1a, 2a).

Further alteration in membrane characteristics, chlorophyll degradation, cell viability and free radical production on exposure to high or low temperature substantiate the earlier reports (Siripornadulsil et al. 2002; Senthil-Kumar et al. 2003, 2007) that responses to these can be elucidated in *Chlamydomonas*. *Chlamydomonas* has also been shown to respond UV stress both transcriptionally and post-transcriptionally (Lilly et al. 2002). UV-A inhibited alternative respiration and the effect of UV-B and UV-C on respiration rate has been clearly demonstrated in *Chlamydomonas* (Mulley et al. 2001). In the present study also, UV-B affected the cell growth (Fig. 2b), in addition to higher chlorophyll degradation (data not shown). Our results clearly demonstrated that acclimation response is seen in all the stresses studied and therefore the relevance of target gene can be efficiently assessed in this system.

Genetic transformation and expression of *codA* gene in *Chlamydomonas*

Although bombardment (Kindle et al. 1989) with microparticles, agitation with glass beads (Kindle 1990), or electroporation (Shimogawara et al. 1998; Siripornadulsil et al. 2002) are widely used methods to introduce DNA in *Chlamydomonas*, *Agrobacterium*-mediated nuclear transformation of *Chlamydomonas* was demonstrated by transforming reporter genes like *gus* and green fluorescent protein (*gfp*) and a few polyamine biosynthetic genes (Kumar et al. 2004, 2005). Because cell wall-less mutants are not required, this technique might have advantage in stress studies. Our study also demonstrated stable integration of *gus* gene and its expression in *Chlamydomonas* by *Agrobacterium*-mediated transformation (Supplementary Fig. 2a–c). Further PCR and dot blot analysis clearly showed the efficient transformation of *codA* gene (Fig. 4a, b) in *Chlamydomonas*. Southern analysis revealed that *Agrobacterium*-mediated transformation of this gene produced single as well as double copy insertions in the transgenics (Fig. 4c). Also higher accumulation of *codA* transcripts in the transgenic lines studied indicated that the gene was efficiently transcribed (Fig. 4d). However, *Chlamydomonas* has historically been considered difficult to transform with foreign DNA. This problem was overcome primarily by using constructs based on native *Chlamydomonas* specific promoters and 3′ flanking regions. To achieve reliable expression of reporter genes, Fuhrmann et al. (1999) resorted to resynthesizing the GFP and Renilla luciferase genes using a *Chlamydomonas* specific codon bias. Selectable antibiotic resistance markers derived from bacterial origin with similarly GC-rich genomes were used to achieve gene expression.

In our studies although we transformed plasmids with CaMV 35S promoter, not customized for *Chlamydomonas*, the *gus* and bacterial *codA* gene (GC rich) integration and expression was efficiently achieved. Even recent studies of Kumar et al. (2004, 2005) had demonstrated stable expression of target gene driven by CaMV 35S in *Chlamydomonas*. In depth analysis of transformants and further probing experiments are necessary to find out the reason for this success. In addition, silencing of introduced genes is a recurrent problem in *Chlamydomonas* transformation and the exact mechanism is not understood. However, in the present study, higher expression of *codA* transcripts and antibiotic resistance of our transgenics ruled out the possibilities of silencing. In fact Ohnishi and Murata (2006) have shown *codA* expression and synthesis of glycinebetaine using the same *codA* gene construct in *Synechococcus* system.

Chlamydomonas transformants expressing *codA* showed stress tolerance

Functional significance of several stress responsive genes like LEAs, SOD, APX, HSPs and several osmolytes like proline, mannitol, glycinebetaine and trehalose has been well documented by overexpression studies. Glycinebetaine was also demonstrated as the most effective osmoprotectant (reviewed in Bartels and Sunkar 2005). However, plant species differ in accumulation of osmolytes and only very few species accumulate glycinebetaine. Glycinebetaine provides protection to cellular machinery under many abiotic stresses (Ohnishi and Murata 2006; Park et al.

2006), mainly by stabilizing the quaternary structure of complex proteins and adjusting the osmotic potential in their cytoplasm to maintain water content. During photosynthesis, it stabilizes both PSII complexes and RuBisCO during salinity and temperature stresses (Papageorgiou and Murata 1995). A non-accumulator of glycinebetaine could be a better system to study the relevance of genes involved in glycinebetaine biosynthesis like the *codA*. Nucleotide BLAST analysis of few known glycinebetaine biosynthetic genes from plants with the *Chlamydomonas* sequences (http://compbio.dfci.harvard.edu/tgi/) indicated no significant nucleotide homology and hence *Chlamydomonas* genome may not have genes for the biosynthesis of glycinebetaine. Further the levels of glycinebetaine in WT *Chlamydomonas* suggest that it may not be a natural accumulator of glycinebetaine (Fig. 4e).

The codA pathway clearly has an advantage over other pathways in glycinebetaine non-accumulators because the substrate choline is directly converted to glycinebetaine in a single step. In addition, this protein does not require any co-factors for catalysis (Sakamoto and Murata 2001). Like many other vascular plants, *Chlamydomonas* might also possess the substrates required for synthesis of glycinebetaine when *codA* is expressed. Our results provided adequate evidences that the *codA* transformants had enhanced *codA* transcript levels and glycinebetaine accumulation. In contrast, expression studies using gene specific primers of *codA* and Southern analysis using *codA* specific probes did not reveal the presence of *codA* gene in WT *Chlamydomonas*.

The relevance of *codA* in imparting tolerance to diverse stresses has been well elucidated. Hayashi et al. (1997) showed that *Arabidopsis* plants expressing *codA* were tolerant to NaCl stress and the plants had higher PSII activity. The unicellular phototrophs like *Synechococcus* were shown to acquire salt tolerance when the *codA* was expressed (Sakamoto and Murata 2001). In fact understanding of the physiological significance and mode of action in vivo, of glycinebetaine in stress tolerance of photosynthetic organisms came from *codA* expressing *Synechococcus* (Deshnium et al. 1995; Nomura et al. 1995; Ohnishi and Murata 2006). Glycinebetaine was shown to impart high temperature tolerance (Papageorgiou and Murata 1995) in tobacco (Yang et al. 2005) transgenics expressing *codA*. The growth rate of the transformants was also maintained under cold stress and high light stress. Earlier studies in maize (Chen et al. 2000), tomato (Park et al. 2006) and *Arabidopsis* (Hayashi et al. 1997) demonstrated that transformants expressing *codA* showed higher cold tolerance and only marginal stress effects. In an earlier study (Alia et al. 1999) also *Arabidopsis* expressing *codA* showed better tolerance to high light stress. Deshnium et al. (1997) had shown that the *Synechococcus* cells expressing *codA* were tolerant to high light-induced stress.

The present study also demonstrated that the *Chlamydomonas* transformants expressing *codA* showed higher levels of tolerance to diverse stresses (Supplementary Fig. 4a; Table 1). The cells grown under 150 mM NaCl showed increased growth and more cell viability under stress. In addition the WT cells enriched with glycinebetaine showed higher growth when subsequently exposed to NaCl-mediated salinity stress. Even when exposed to PEG-mediated osmotic stress, *codA* transformants showed higher growth, less chlorophyll degradation and also maintained high cell viability (data not shown). Although temperature tolerance of the *codA* transformants is marginal, these cells showed relatively higher survival and growth rates when exposed to high light stress with methylviologen (Supplementary Fig. 4).

The relevance of *codA* was seen by exposing the transformants directly to severe stress levels in other studies, which to some degree provides information on basal level tolerance. There are evidences to show that several transgenics expressing stress responsive genes did not show elevated levels of tolerance over wild type following acclimation (Prandl et al. 1998; also reviewed in Senthil-Kumar et al. 2007). These studies confirm that the acclimation response of the transgene will alone provide clues about its relevance. In this study, the importance of *codA* gene in stress tolerance was demonstrated in the acclimated conditions under the background of expression of many other stress responsive genes. Hence the higher tolerance levels shown by *codA* transgenics is unique and distinct from other studies wherein the stress effects were studied under non-acclimated conditions.

In summary, the results reported in this study clearly demonstrated that functional significance of stress responsive genes for basal and acquired tolerance can be studied by over-expressing the gene of interest in *Chlamydomonas*. Responses to diverse stresses make it an ideal system for characterization of complex stress transcriptomes of higher plants.

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