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SHORT COMMUNICATION

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Cyanobacterial Mn-catalase 'KatB': Molecular link between salinity and oxidative stress resistance

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ABSTRACT

Catalases are ubiquitous enzymes that detoxify H_2O_2 in virtually all organisms exposed to oxygen. The filamentous, nitrogen-fixing cyanobacterium, *Anabaena* PCC 7120, shows the presence of 2 genes (*katA* and *katB*) that encode Mn-catalases. We have recently shown that pre-treatment of *Anabaena* with NaCl causes substantial induction of the KatB protein, which consequently leads to increased oxidative stress resistance in that cyanobacterium. Interestingly, when compared to the wild-type, the *katB* mutant shows decreased growth and impaired photosynthetic activity in the presence of NaCl. Furthermore, the NaCl-treated *katB* mutant is extremely sensitive to H_2O_2. In this study, the ultrastructural changes occurring in the *katB* mutant and the wild-type *Anabaena* cells are analyzed to understand the cellular basis of the above-mentioned protective phenomena. Other data show that a wide variety of osmolytes induce *katB* expression in *Anabaena*, indicating that *katB* is a genuine osmo-inducible gene. These results have important biotechnological implications for the development of novel cyanobacterial biofertilzers and transgenic plants with improved resistance to salinity.

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The presence of oxygen is a double-edged sword for aerobes. The transfer of electrons through the respiratory chain to oxygen, that drives the production of ATP, also results in the concomitant generation of the deleterious reactive oxygen species (ROS) such as the superoxide radical ($O_2\cdot$), hydrogen peroxide (H_2O_2) or the hydroxyl radical ($\cdot OH$).[1] In living systems, H_2O_2 forms the veritable connection between $O_2\cdot$ and $\cdot OH$. Although, H_2O_2 is produced directly by several oxidases, the dismutation of $O_2\cdot$ by superoxide dismutase (SOD) is the major source of H_2O_2 production in cells.[2] However, though not very reactive by itself, H_2O_2 can very quickly become the key ROS that imperils cell's survival. In the presence of iron, an essential micronutrient, H_2O_2 reacts with Fe^{2+} to generate the $\cdot OH$, which can damage all the cellular macromolecules at diffusion-controlled rates.[3] Thus, decomposition of H_2O_2 is very pertinent for cellular existence.

Detoxification of H_2O_2 is primarily brought about by peroxidases and catalases. Peroxidases (e.g. ascorbate peroxidase, peroxiredoxins, etc.) require electron-donating reducing agents as accessories to decompose H_2O_2; whereas catalases directly detoxify H_2O_2 by a dismutation reaction, forming H_2O and molecular oxygen in the process.[4] Catalases, essentially are of 2 types viz; heme catalases or manganese (Mn)-catalases. The typical monofunctional catalase and the catalase-peroxidase (i.e. KatG) contain heme whereas Mn-catalases lack heme, but contain Mn at their active site.[5,6] The heme catalases are widespread in both eukaryotes and prokaryotes while Mn-catalases are found exclusively among prokaryotes and archea.[5]

Cyanobacteria, widely regarded as the progenitors of plant chloroplasts, were responsible for the early oxygenation of Earth's atmosphere.[7] Due to their close association with O_2, they are likely to have developed multiple stratagems to surmount the toxic effects of ROS. Moreover, the nitrogen-fixing strains of cyanobacteria remain the only form life that can harvest solar energy to fix atmospheric nitrogen.[8] Incidentally, naturally occurring, filamentous, nitrogen-fixing cyanobacteria (e.g., *Anabaena*) are widely used as biofertlizers in the paddy fields of Southeast Asia.

Over the last few years, our laboratory has focused on dissecting the mechanisms responsible for overcoming oxidative stresses in the filamentous, heterocystous, nitrogen-fixing cyanobacterium, *Anabaena* PCC 7120.[9-13] This cyanobacterium contains an arsenal of ROS detoxifying enzymes, which include SODs, peroxiredoxins (Prxs), catalases etc.[14] Along with the presence of several genes encoding peroxiredoxins (Prxs), this *Anabaena* has 2 genes that encode Mn-catalase (*alr0998* i.e., *katA*, and

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alr3090 i.e. *katB*). In spite of the presence of genes encoding Mn-catalases, no detectable catalase activity is observed in the control (i.e., unstressed) or H_2O_2-treated *Anabaena*.[10] In comparison, Prxs like *all541* (encoding All1541) and *alr4641* (encoding Alr4641) are found to be transcriptionally induced in response to H_2O_2 or methyl viologen. Additionally, enhanced synthesis of the All1541 and Alr4641 proteins is observed when *Anabaena* is subjected to oxidative stress.[9,11] These observations apparently suggest that, rather than catalases, Prxs may be the primary proteins that detoxify H_2O_2 in *Anabaena*. However, despite this distinct induction, endogenous production of Prxs is unable to rescue *Anabaena* from the deleterious effects brought about by exposure to H_2O_2. In fact, treatment with just 0.5 – 1 mM H_2O_2 leads to cell lysis/death in *Anabaena*.[13]

Surprising results with wide ramifications for oxidative stress resistance were obtained while performing cross-protection experiments with *Anabaena* PCC 7120. *Anabaena* cells that were pre-treated with NaCl showed high degree of resistance to H_2O_2 and could withstand exposure to as high as 3 mM H_2O_2.[13] Keeping in mind the presence of *katA*/*B* genes in *Anabaena* and catalase being the known 'classical' enzyme that protects organisms from H_2O_2, catalase activity of the above-mentioned cultures was assessed on zymograms. Interestingly, the salt-treated *Anabaena* cells showed the presence of a distinct catalase activity. When probed with the KatA or KatB antiserum (on native Western blots), the zone of catalase activity matched exactly with the signal obtained from the KatB antiserum, demonstrating that KatB was the catalase induced in response to salt stress in *Anabaena* PCC 7120.[13]

Unlike the wild-type *Anabaena* PCC 7120, the NaCl-treated *katB* mutant was found to be particularly sensitive to H_2O_2, and exposure to H_2O_2 caused increased formation of lipid peroxides, oxidized proteins and total peroxides in the *katB* mutant.[13] In fact, a considerably higher level of oxidized proteins was found in the culture medium of the mutant at the end of 24 h (Fig. 1A). Under the transmission electron microscope, distinct structural changes were observed between the wild-type cells and the *katB* mutant (Fig. 2). The wild-type cells appeared to be robust and showed proper thylakoid integrity even after exposure to the oxidizing agent. In contrast, the *katB* mutant lost the thylakoid ultrastructure, and in some cells, loss of cellular contents (indicative of lysis) was also observed. Carboxysomes were observed in the wild-type cells whereas no such structures were seen in the *katB* mutant. In filamentous cyanobacteria, the content of chlorophyll *a* is a good indicator of growth/cellular integrity and this parameter

Figure 1. Detection of oxidized proteins. Proteins were extracted by TCA precipitation from the culture medium of NaCl-treated wild-type *Anabaena* (WT) or the *katB* mutant (*katB*$^{-}$) cells after exposure to H_2O_2 (1mM). These proteins were derivatized with dinitrophenol (DNP), resolved on SDS-PAGE and transferred to nitrocellulose membrane. Subsequently, these proteins were probed with the monoclonal DNP antiserum. A Ponceau S-stained part of the blot is shown in the lower panel as loading control. The oxidized proteins were detected as mentioned in the OxyBlot oxidized protein detection kit (Thermo Scientific, 23280).

is routinely used to assess resistance to various stresses. Hence, to further substantiate the TEM data, the chlorophyll *a* content of the NaCl-treated wild-type or the *katB* mutant was monitored. After 24 h, drastic reduction (~10-fold) in the chlorophyll *a* content was observed in the *katB* mutant whereas only around 10% decrease was observed in the corresponding wild-type. Clearly, the induced KatB protein detoxified H_2O_2, consequently ameliorating its toxic effects to a large extent in the wild–type strain, whereas the deleterious effects of H_2O_2 were obvious in the *katB* mutant.

The *katB* promoter-*gfp* fusion construct[13] was gainfully employed to determine the various osmotic stimuli that could activate *katB* expression. All the osmolytes tested, i.e. NaCl, sucrose, glycerol, manitol, sorbitol, and PEG, were able to activate the *katB* promoter in *Anabaena* (Fig. 3). Induction of this promoter with ionic osmolytes (NaCl) as well as non ionic cell permeable osmolytes, suggests that *katB* is indeed an osmotically induced gene. Unexpectedly, KatB is not induced with its own substrate, hydrogen peroxide. Desiccation, an

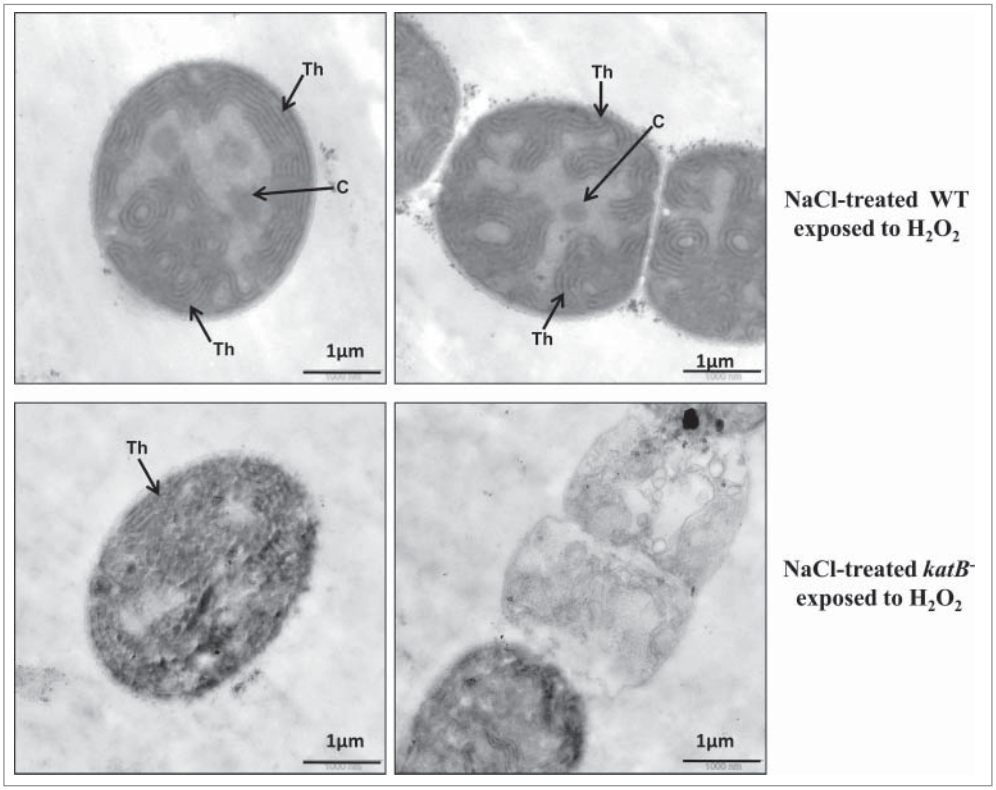

Figure 2. Ultrastructural features of the NaCl-treated wild-type *Anabaena* (WT, upper panel) or *katB* mutant (*katB*$^-$, lower panel) after exposure to H_2O_2 (for 24 h) as seen under the transmission electron microscope. Samples were processed for transmission electron microscopy as described earlier.[22] Thylakoid membranes (Th) and carboxysomes (C) are indicated. Severely disintegrated thylakoid membranes and a distinct loss of ultrastructure are evident in the *katB* mutant filaments exposed to H_2O_2.

extreme form of osmotic stress, also induces synthesis of KatB in *Anabaena*. Also, *katB* expression is regulated by the nitrogen status of the medium and there is a distinct lack of *katB* transcription in heterocysts (cells that fix nitrogen).[13] Thus mechanism underlying the transcriptional activation of *katB* is complicated and several environmental cues are integrated in this process. The relatively long (~450-bp) regulatory region between the *katB* promoter and the translational start of KatB is likely to provide ample space for various factors to bind *in trans* and modulate expression. In the unicellular cyanobacterium, *Synechocystis* PCC 6803, histidine kinases are known to regulate several osmotically induced genes.[15,16] The presence of these homologs in *Anabaena* lends support to the idea that *katB* too may be regulated by proteins such as histidine kinases.

Our study has provided new insights into the enhancement of cyanobacterial salt tolerance by combined nitrogen that was reported from our laboratory over 25 y ago.[17] In that study, the inhibition of Na^+ influx by combined nitrogen was suggested to be a major mechanism for overcoming salt stress in *Anabaena*. Salt is known to increase ROS (e.g. H_2O_2) in several organisms including *Anabaena*.[13,18] Interestingly, our recent findings show that the presence of combined nitrogen enhances production of KatB in *Anabaena*.[13] Consequently, the increased levels of KatB are likely to help *Anabaena* overcome the oxidative effects of salt stress. So, along with the inhibition of Na^+ influx, reduction in

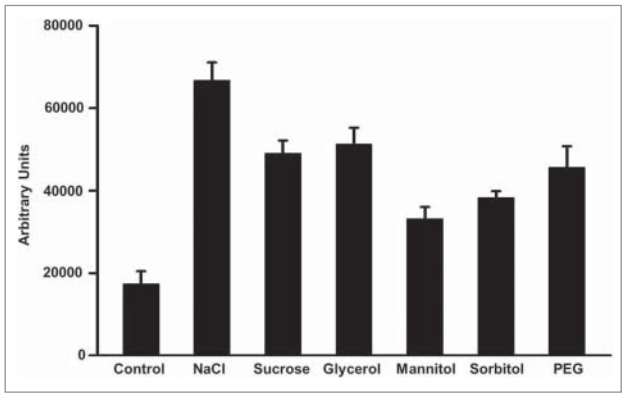

Figure 3. The *katB* promoter-*gfp* fusion construct was transformed into *Anabaena* PCC 7120 and the *katB* promoter activity was monitored[13] in the presence of various osmolytes such as NaCl (150 mM), sucrose (300 mM), glycerol (300 mM), manitol (300 mM), sorbitol (300 mM), and PEG (100 mM). Cells were exposed to the above-mentioned osmolytes for 18h. The green fluorescence (λ_{ex} = 490 nm, λ_{em} = 520 nm) of the reporter GFP is plotted as bar diagram. Standard deviation for 5 independent experiments is shown as error bars.

the levels of ROS brought about by KatB may also contribute to the salinity tolerance of *Anabaena* observed under these conditions.

As far as the future prospects are concerned, 2 lines of research appear to be particularly appealing. One, off course, is to have a detailed understanding of how the *katB* gene is regulated in *Anabaena*. The other aspect has more practical applications i.e., can the KatB-overexpressing *Anabaena* be employed as a more efficient biofertilzer or can the *katB* gene transferred to plants in the hope of improving their stress resistance? Once synthesized, KatB persists in cells for several days even when the inducing stimuli (e.g., NaCl) is removed.[13] Thus, *Anabaena* can be exposed to NaCl, which can be washed off subsequently. These natural, non-recombinant, KatB-overproducing strains could possibly work as more efficient biofertilizers than their normal unstressed counterparts. In plants, overexpression of catalase is directly correlated with improved resistance to salinity. For example, heterologous expression of *E. coli* KatE (a KatG-type catalases) led to a considerable increase in the ability of rice to with stand salt stress.[19] However, it should be noted that many heme catalases lose their activity when the temperature rises above 40°C.[10,20] In comparison, KatB has been shown withstand temperatures over 80°C and remain functional over a wide range of pH or salt concentrations.[13,21] In our opinion, due to its more robust nature, KatB appears to be a promising candidate for transfer to crop plants in order to improve their resistance to various environmental stresses. All these aspects are currently being explored in our laboratory.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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