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# A novel plant glutathione peroxidase-like protein provides tolerance to oxygen radicals generated by paraquat in *Escherichia coli*

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**Abstract**

Citrus salt-stress associated protein (Cit-SAP) reveals significant sequence homology to mammalian glutathione peroxidase (GP). In an attempt to assign biological function to this protein, transformed *E. coli* cells expressing Cit-SAP were examined for their ability to tolerate free radicals formed by paraquat, an $O_2^-$ radical forming agent. In the presence of paraquat, the survival rate of the transformed bacteria expressing Cit-SAP was much higher as compared to the wild-type bacteria. The results support the assumption that Cit-SAP is a plant GP-like protein which participate in the enzymatic system aimed at scavenging oxygen free-radicals in plants.

*Key words:* Glutathione peroxidase; Oxidative stress; Citrus; Salt tolerance

## 1. Introduction

In animal cells, glutathione peroxidase (GP) plays a key role in the defense against the oxidative damage caused by $H_2O_2$ or lipid peroxides [1]. These free radicals are formed as by-products in energy-generating organelles [2,3] and in various stress conditions such as ionizing radiation and redox cycling agents. In *E. coli*, superoxide dismutase (SOD) and catalase are key enzymes in the major cell machinery responsible for scavenging oxygen free radicals. GP activity in *E. coli* was not yet found [4], although it was claimed to be present in several other prokaryotic organisms [5]. The presence of GP and its potential physiological role in photosynthetic organisms, particularly in plants, is not yet clear. However, growing evidence for its presence and activity in blue green cyanobacteria [6], chlamydomonas [7], marine diatoms [8], *Euglena* [9], moss [10], and leaves of several higher plants [11], indicates that this enzyme might be widely spread among photosynthetic organisms and might have a role in their defence against free radicals. Recently [12], a cDNA with a significant homology to animal GP and to the bacterial gene *btuE* was cloned from tobacco protoplasts. This gene was suggested to be controlled by oxidative stress, caused by the removal of the plant cell wall or by the chemicals involved in the protoplast generating treatment. However, the actual expression of the corresponding protein was not demonstrated, nor its specific enzymatic activity. Independently to this study, a gene, designated *csa*, which reveals a high degree of homology (~ 86%) to the tobacco gene, was isolated from Shamuti orange cultured cells that were acclimated to high levels of NaCl [13]. The protein product of *csa*, a citrus salt-associated protein (Cit-SAP), was found to be present in all citrus plants tested, and its level is increased in cultured cells exposed to NaCl and in citrus plants upon irrigation with salt [14]. The homology of Cit-SAP to mammalian GP, and the increase in its level due to irrigation with elevated concentrations of NaCl, suggest that NaCl treatment may generate free radicals which mediate increase in the levels of glutathione peroxidase. Until now, studies have indicated that the hydrogen peroxide scavenging systems in plants consist of other peroxidases, in particularly ascorbate peroxidase [15,16]. The elevated levels of Cit-SAP in response to NaCl treatment may indicate that this particular protein, which is induced under restricted physiological conditions such as salt stress [14], plays a role in scavenging excess of $H_2O_2$ and lipid peroxides [10]. Our efforts to demonstrate that $CSA^+$ cells have GP activity have not yet succeeded. This may be due to differences observed between the primary structures of Cit-SAP and mammalian GP [13], which probably effect the stability, specificity or the conditions required for the activity of Cit-SAP as compared to GP.

As an alternative tool to study the function of Cit-SAP and assess its putative activity as a hydrogen perox-

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*Abbreviations:* GP, glutathione peroxide; SOD, superoxide dismutase; Cit-SAP, citrus-salt-stress associated protein; $CSA^-$, *E. coli* strain DH5α; $CSA^+$, *E. coli* strain DH5α transformed with pARO1 plasmid which contains *csa*.

ide scavenger, we have used *E. coli* as a heterologous model system. In accord with the assumption that Cit-SAP is a glutathione peroxidase, we demonstrate in this study that *E. coli* which express the protein product of *csa*, are much more tolerant to the free radical generating agent paraquat.

## 2. Experimental

### 2.1. Plant material

Adapted salt-tolerant cell lines derived from Shamuti orange (*Citrus sinensis* L. Osbeck) were used [17].

### 2.2. E. coli *strain and plasmid*

*E. coli* strain DH5α was used in this study. pARO1 plasmid (Fig. 1) contains a full-length cDNA clone which corresponds to the *csa* gene that encodes for Cit-SAP; the gene was placed under the control of the *lac* promoter in the Bluescript SK⁻ vector (Stratagene) [13]. Cells transformed with pARO1 are designated as CSA⁺ cells, while cells transformed with only Bluescript SK⁻ vector are designated as CSA⁻ cells.

### 2.3. Growth media and conditions used for selection on paraquat

The citrus cells were grown in liquid Murashige and Tucker medium as described [18]. *E. coli* cells were grown in LB medium in the presence of ampicilline (50 μg/ml), at 37°C with shaking. For examination of the viability of CSA⁺ and CSA⁻ in the presence of paraquat, 20 ml of *E. coli* cells were grown to $A_{600}$ = 0.8 or to $A_{600}$ = 3. Aliquots (100 μl) were taken from these cultures and diluted in LB to an estimated number of 200–300 colonies per plate. Two aliquots were spread from the appropriate concentration on LB-agar plates (5 cm in diameter), containing 0.1, 0.15 and 0.2 mM of paraquat (methylviologen, 1,1′-dimethyl-4,4′-bipyridinium dichloride from Sigma) and 50 μg/ml ampicillin. After spreading, cells were grown at 37°C. Tolerant colonies usually appeared after 40–48 h.

### 2.4. Cell disruption and superoxide dismutase assay in E. coli

Prior to the plating of *E. coli* as described above, cell samples were washed and sonicated in 50 mM phosphate buffer (pH 7.8) containing 1 mM EDTA and isoascorbate (14 mg/ml). The amount of the protein was determined by the method described by Bradford [19], and enzyme activity on 10% polyacrylamide slab gels was visualized using the method of Beauchamp and Fridovich [20].

### 2.5. Gel electrophoresis

Protein extract of CSA⁺ was obtained by sonication of the bacteria in 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, followed by the removal of the non-soluble debris by centrifugation; for two-dimensional PAGE, the supernatant was diluted with the isoelectric-focusing sample buffer [21]. Protein extract of citrus cultured cells, as well as two-dimensional PAGE, were carried out as described by Ben Hayyim et al. [14]. Western blots were analyzed by reactions with anti-Cit-SAP antibodies preadsorbed with crude extract of *E. coli* and staining with alkaline phosphatase conjugated to a second antibody.

## 3. Results

### 3.1. Expression of Cit-SAP in E. coli and Shamuti orange callus

Two-dimensional Western blots of protein extracts obtained from CSA⁺ and adapted salt-tolerant citrus cells, detected by anti Cit-SAP antibodies, are presented in Fig. 2. These blots indicate that although a protein of the same $M_r$ is expressed in both *E. coli* and the plant, as was previously shown [13] by one-dimensional SDS-PAGE, subtle differences between the *E. coli* and the plant protein do exist. In both types of cells, two protein forms having the same $M_r$ but different pI values were detected by the anti Cit-SAP specific antibodies. Both the plant and the transformed bacteria, share a common protein spot of pI = 6.1 (Fig. 2C, indicated by an arrow), but differ in the pI value of their second protein. In the plant cells (Fig. 2A), the second protein form is more acidic than the common spot and much weaker. This spot was detected when the two-dimensional PAGE was highly loaded with protein and the blot was detected by using the alkaline phosphatase method. In CSA⁺ cells, the second protein form is more basic and more intense than the common spot of the protein with pI = 6.1 (Fig. 2B).

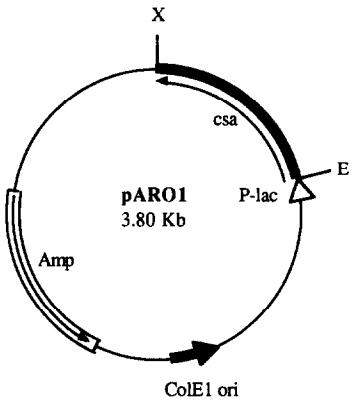


Fig. 1. Schematic presentation of pARO1, a plasmid that contains the *csa* gene and express the corresponding Cit-SAP protein. A 0.8 kb *Eco*RI-*Xho*I fragment that contains the cDNA of the *csa* gene and its flanking regions (Holland et al., 1993) is placed under the *lac* promoter of blue script SK-vector (Stratagene). X = *Xho*I restriction site; E = *Eco*RI restriction site. P-lac = *lac* promoter.

### 3.2. Phase-dependent expression of SOD and Cit-SAP in E. coli

Since SOD catalyses the production of $H_2O_2$, the substrate of GP, from $O_2^-$ radicals, this enzyme activity was determined for both CSA⁺ and CSA⁻ taken from cultures at $A_{600}$ = 0.8 and $A_{600}$ = 3. As shown in Fig. 3, the profile of SOD bands originating from late log phase cells is different from that of stationary phase cells. The band corresponding to Fe,SOD (as judged by sensitivity to $H_2O_2$) is stronger in the stationary phase cells. On the other hand, late log phase cells contain an additional band (probably a hybrid between the Fe,SOD and the Mn,SOD). No difference was observed between the intensity of the SOD bands of CSA⁺ and CSA⁻. Stationary phase CSA⁺ cells also display much higher levels of Cit-SAP as compared to late logarithmic phase cells (Fig. 3).

### 3.3. Contribution of csa transformation to paraquat tolerance in E. coli

CSA⁺ and CSA⁻ cells, at different growth phases, were assayed for their ability to grow on LB-agar plates containing increasing paraquat concentrations. As shown in

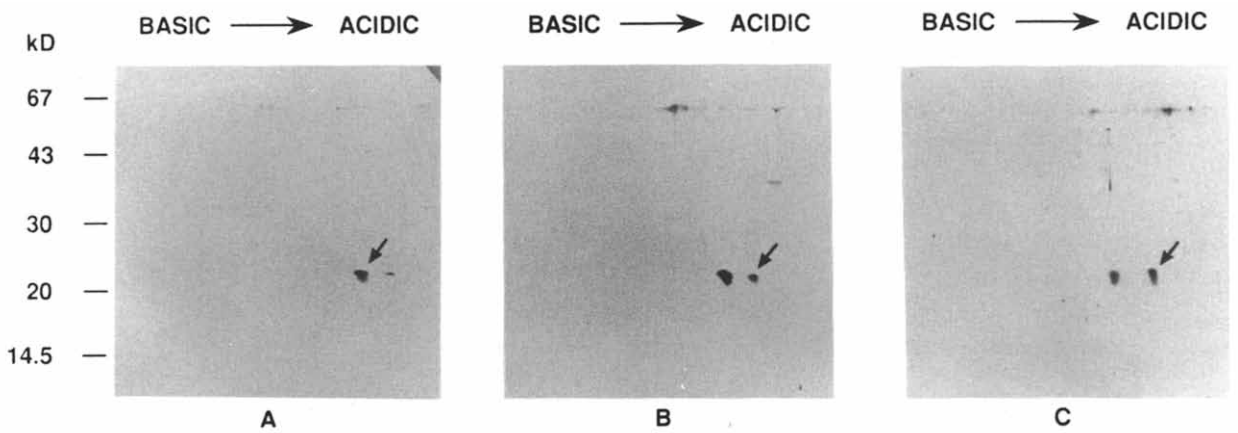


Fig. 2. Two-dimensional gel electrophoresis of proteins (250 μg) extracted from: (A) adapted salt-tolerant cell line derived from Shamuti orange, grown in the presence of 200 mM NaCl; (B) late log phase CSA$^+$ *E. coli* cells containing the *csa* gene; (C) mixture of extracts A (125 μg) and B (125 μg).

Fig. 4, both types of cells exhibited more tolerance to paraquat when plated at their stationary phase ($A_{600}$ = 3.0), as compared to the late log phase ($A_{600}$ = 0.8). However, in all cases, CSA$^+$ cells were more viable than CSA$^-$ cells when grown in the presence of paraquat. The most distinctive effect was observed when the cells were spread at $A_{600}$ = 0.8 on plates containing 0.1 mM paraquat. Under these conditions, the number of CSA$^+$ cells that formed colonies exceeded the number of CSA$^-$ cells by a factor of 5 (Fig. 4A).

## 4. Discussion

The expression of the citrus *csa* gene in *E. coli* clearly confers higher tolerance to paraquat. The most pronounced effect of *csa* was observed when cells from cultures at $A_{600}$ = 0.8 were spread on plates containing 0.1 mM paraquat. Under these conditions the number of *csa* transformed cells that were tolerant to paraquat exceeds the number of non-transformed control cells by a factor of 5. The tolerance to paraquat depends on the growth stage from which the bacteria were used for spreading the cells on the plates. Hence, cells that were spread from $A_{600}$ = 0.8 cultures are less tolerant than cells spread from cultures at $A_{600}$ = 3.0. This increased tolerance does not depend on *csa*, since it occurs in both CSA$^+$ and CSA$^-$ cells. Yet, even when this intrinsic *E. coli* tolerance is increased, the effect of *csa* is still well detected. This effect is additive to the activity of the intrinsic free-radicals scavenging system of the bacteria, also in the stationary phase. Increased Mn,SOD levels in response to glucose exhaustion [22] and to paraquat [23,24] have been previously reported. If Cit-SAP acquires GP-like activity, then its substrate might be $H_2O_2$ in addition to other peroxides. The concentration of $H_2O_2$ depends on the activity of SOD. In the presence of paraquat, high flux of $O_2^-$ is generated and should be dismutated to hydrogen peroxide by the action of SOD in order to be available for GP. Therefore, Cit-SAP activity will reach its full potential when SOD level is high enough. We found that a protein band that corresponds to Fe,SOD activity is stronger in stationary cells than in the end of the log phase cells. Perhaps, this form of SOD is more efficient in dismutation of $O_2^-$ under the physiological conditions imposed at the stationary phase and may be responsible for the higher tolerance to paraquat displayed by stationary phase cells.

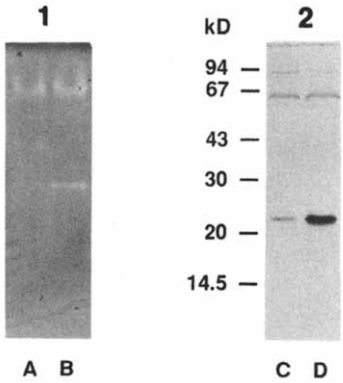


Fig. 3. Superoxide dismutase activity and Cit-SAP levels of CSA$^+$ cells. (1) PAGE presenting SOD activity at the late log phase (A) and at the stationary phase (B). (2) Western blot analysis comparing the levels of Cit-SAP in CSA$^+$ at the late log phase (C) and at the stationary phase (D).

We demonstrated previously [13] that *E. coli* transformed with *csa* synthesize a protein with a similar $M_r$ to Cit-SAP. In the present study we have further investigated the identity of the bacterial and plant proteins by two-dimensional PAGE analysis. This analysis (Fig. 2) indicates that citrus and *E. coli* synthesize several forms of the protein with different pI values. In addition to a common protein that is shared by the two organisms (pI = 6.1), the *E. coli* contains an additional form of Cit-SAP with a more basic pI. This protein is the major Cit-SAP form that is synthesized in *E. coli*. Plants, on the other hand, express a different form of Cit-SAP which is more acidic and is minor compare to the form common to the bacteria and plant. These findings suggest that the *csa* encoded protein may be modified differently in citrus

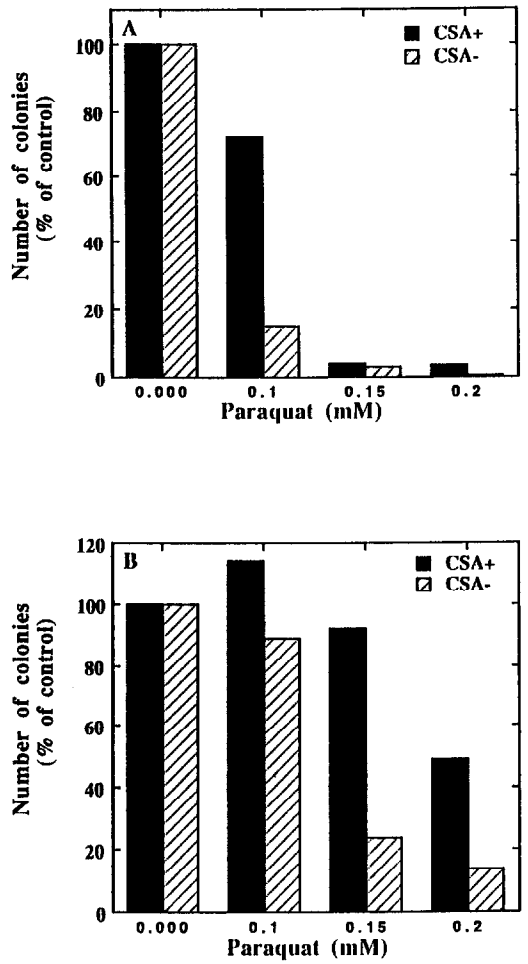


Fig. 4. Viability of $CSA^+$ and $CSA^-$ in the presence of paraquat. The bacterial cells were grown to $A_{600} = 0.8$ (A) and $A_{600} = 3.0$ (B) in the absence of paraquat, and aliquots were spread on plates containing 50 μg/ml ampicillin and different concentrations of paraquat. $CSA^+$ cells contain plasmid pARO1; $CSA^-$ cells contain control vector plasmid bluescript SK-(Stratagene). For experimental details see text.

and in *E. coli*. The main protein product of the plant however is also found in *E. coli* although its proportion is much more reduced. We do not yet know the differences in the chemical nature of the three forms of Cit-SAP, nor the level of similarity of their biochemical activity. These structural differences may be due to post-transcriptional modifications or changes in the amino acid backbone of the protein.

Our analyses indicate, that stationary phase cells contain higher levels of *csa* as compared to end of the log phase cells. We do not know whether the reason for this increased expression is a higher induction of the *lac* promoter or because stationary phase cells accumulate more copies of the plasmid containing the *csa* gene. The increased level of Cit-SAP in stationary phase cells may indicate that Cit-SAP expression in cells under these physiological conditions is of advantage to the cells. Endogenous GP activity have not been demonstrated in *E. coli* [4]. The increased tolerance to paraquat of $CSA^+$ cells is the first indication that Cit-SAP is possibly an antioxidant enzyme. The fact that *csa* is very homologous (86%) to the evolutionary distinct tobacco gene, and that Cit-SAP was found to be expressed in other plant species (data not presented), suggests that this protein is highly conserved in plants, and probably plays an important role in controlling the level of oxygen radicals. The present findings that a plant GP-like protein is active in *E. coli*, may facilitate our ability to use the *E. coli* heterologous system for the selection of plant or human GP mutants with higher efficiencies to scavenge $H_2O_2$ or lipid peroxides. Such mutants could be generated by randomly mutagenizing the *csa* gene in vitro and subsequently transforming *E. coli* with the mutated DNA followed by selection for colonies that display higher tolerance to paraquat.

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## References

[1] Kappus, H. and Sies, H. (1981) Experimentia 37, 1233–1258.
[2] Wendel, A. (1980) in: Enzymatic Basis of Detoxification (Jacoby, W.D. ed) pp. 333–353, Academic Press, New York.
[3] Halliwell, B. and Gutteridge, J.M.C. (1985) Free radicals in Biology and Medicine, Oxford University Press, Oxford.
[4] Smith, J. and Schriff, A. (1979) Comp. Biochem. Physiol. 63B, 39–42.
[5] Neubert, D., Rose, T. and Lehninger, A.L. (1962) J. Biol. Chem. 237, 2025–2031.
[6] Tozum, S.R.D. and Gallon, J.R. (1979) J. Gen. Microbiol. 11, 313–316.
[7] Yokota, A., Shizeoka, S., Anishi, T. and Kitaoka, S. (1988) Plant Physiol. 86, 649–651.
[8] Price, M.N. and Harrison, J.P. (1988) Plant Physiol. 86, 192–199.
[9] Overbauch, J.M. and Fall, R. (1985) Plant Physiol. 77, 437–442.
[10] Dhindsa, R.S. (1981) Plant Physiol. 95, 648–651.
[11] Drotar, A., Phelps, P. and Fall, R. (1985) Plant Sci. 42, 35–40.
[12] Criqui, M.C., Jamet, E., Parmentier, Y., Marbach, J., Durr, A. and Fleck, J. (1992) Plant Mol. Biol. 18, 623–627.
[13] Holland, D., Ben-Hayyim, G., Faltin, Z., Camoin, L., Strosberg, A.D. and Eshdat, Y. (1993) Plant Mol. Biol. 21, 923–927.
[14] Ben-Hayyim, G., Faltin, Z., Gepstein, S., Camoin, L., Strosberg, A.D. and Eshdat, Y. (1993) Plant Sci. 88, 129–140.
[15] Nakano, Y. and Asada, K. (1987) Plant Cell Physiol. 28, 131–140.
[16] Mittler, R. and Zilinskas, B.A. (1991) Plant Physiol. 97, 962–968.
[17] Kochba, J., Ben-Hayyim, G., Spiegel-Roy, P., Saad, S. and Neumann, H. (1982) Z. Pflanzen. Physiol. 106, 111–118.
[18] Ben-Hayyim, G., Kafkafi, U. and Ganmore-Neumann, R. (1987) Plant Physiol. 85, 434–439.
[19] Bradford, M.N. (1976) Anal. Biochem. 72, 248–254.
[20] Beauchamp, B. and Fridovich, I. (1971) Anal. Biochem. 44, 276–287.
[21] Damerval, C., de Vienne, D., Zivy, M. and Thiellement, H. (1986) Electrophoresis 7, 52–54.
[22] Hassan, H.M. and Fridovich, I. (1979) J. Bacteriol. 132, 505–510.
[23] Hassan, H.M. and Fridovich, I. (1977) J. Biol. Chem. 252, 7667–7672.
[24] Greenberg, T.J. and Demple, B. (1989) J. Bacteriol. 171, 3933–3939.