A NUCLEOCYTOPLASMIC SHUTTLING PROTEIN IN OXIDATIVE STRESS TOLERANCE

David W. Ow and Wen Song

Plant Gene Expression Center, USDA/UC Berkeley, Albany, CA 94710

Introduction

The use of plants for the extraction of toxic metals and radionuclides has received much attention as a cost effective remediation technology, where brown fields would turn green with solar driven pumps that suck the pollutants for harvest. The mere fact that brown fields and mine sites are scarcely populated with vegetation, however, shows that plants do not thrive in toxic environments. The inability of plants to germinate or grow past a seedling stage also indicates that plants are quite capable of pollutant uptake, but not of pollutant detoxification. Therefore, a key to the phytoextraction technology is overcoming the inability of plants to detoxify high pollutant content.

Previously, in an attempt to identify genes that would enhance metal stress tolerance, we constructed a cDNA expression plasmid library from *S. pombe, Arabdiposis* and *Brassica juncea* mRNA, transformed the libraries into wild type *S. pombe,* and selected for Cd hypertolerant colonies. This led to the identification of a set of cDNAs capable of enhancing metal stress tolerance [Ow et al, 2000]. Here, we report on the properties of one *S. pombe* gene that confers hypertolerance to cadmium or oxidizing chemicals. Since this gene plays a role in oxidative stress, we named it *oxs1*$^+$.

Material and Methods

Biological Materials. *S. pombe* strain JS23 (*h*$^+$, *ura4.294, leu1.32*) was used as the wild type host. TP108-3C (*h* $^-$, *leu1.32, ura4* $^-$, *pap1::ura4*$^+$) and TP17-13C (*h* $^-$, *leu1.32, crm1-1R*) were provided by Dr. T. Toda (Imperial Cancer Research Fund, London, UK) [Kudo, 1999]. Genetic manipulations were carried out according to the Fission Yeast Handbook available at: http://www.bio.uva.nl/pomb/handbook/. Growth media used were the complete medium YG (1% yeast extract and 2% glucose), the minimal medium SG (0.67% yeast no base and 2% glucose) or the minimal medium EMM2 (Edinburgh minimal medium) for used with the thiamine repressible *nmt1* promoter. Where necessary, 0.02 mg/ml of leucine and/or uracil were included in the minimal media.

Stress tolerance assays. *S. pombe* cells were grown to late log phase, serially diluted in liquid SG medium and 6 ul of each diluted culture (ranging from 10 to 10^6 cells, in ten fold increments) were spotted onto agar medium containing the specific compound at the concentration indicated. The spots were allowed to dry and the plates were incubated at 30° C for 3-5 days.

Recombinant DNA. Standard methods were used throughout for the construction of plasmids and fusions with the enhanced green florescent protein (EGFP) of the pREP41EGFPN vector [Craven et al., 1998]. Northern blots were conducted on total RNA extracted from *S. pombe* as described [Ortiz et al., 1992]. Total RNA from plant samples was extracted using the Qiagene plant RNAeasy kit.

Results & Discussion

Fission yeast gene. With the *oxs1*$^+$ cDNA as a probe, a northern blot of total *S. pombe* RNA detects a single major band. In the hypertolerant strain expressing the *oxs1*$^+$ cDNA from a plasmid, the *oxs1*$^+$ transcript level is significantly higher, which is a positive correlation between overexpression of a gene product and higher stress tolerance. However, it appears that *oxs1*$^+$ transcription is not affected by metal or oxidative stress, as a change in the steady-state transcript level was not detected when wild type *S. pombe* was subjected to Cd (250 uM) or diamide (2 mM) for 30 min, 3 or 6 hr.

Homologous genes are found in the human, mouse, worm, fruit fly and *Arabidopsis* genome databases. To determine if the OXS1 homologue proteins can function in *S. pombe*, the *Arabidopsis OXS1* and the *Drosophila OXS1* cDNAs were inserted into yeast expression vectors and transformed into JS23. Both homologue cDNAs behaved similarly to that of *S. pombe oxs1*$^+$ in enhancing tolerance to Cd, diamide and tert-butyl hydroperoxide (tert-BuOOH) (Figure 1). This indicates that OXS1 proteins have a conserved function among different organisms, and most probably, a role in oxidative stress tolerance.

To gain insight into the cellular location of the OXS1, *S. pombe* was transformed with pWS26, a plasmid producing an EGFP-OXS1 fused protein. An EGFP-EGFP fused protein with a size comparable to the EGFP-OXS1 fusion was used for a control. The EGFP-EGFP protein shows a uniform green signals under fluorescence microscopy in both the cytoplasm and the nucleus (Figure 2a), and this pattern was unaffected by the addition of diamide. In contrast, with the EGFP-OXS1 fusion, the green signals were seen in the cytoplasm, but were excluded from the nucleus (Figure 2b). When the cells were treated with diamide, however, nuclear localization was observed. In approximately half of the cells, fluorescent signals were detected in the nucleus, as well as in the cytoplasm, within 20 minutes after the addition of diamide. By 30 minutes, a strong signal is seen in most cells (Figure 2c). Localization of the signal appeared homogenous in an asynchronous culture, suggesting that OXS1 subcellular location is not affected by the cell cycle. Hence, OXS1 appears to be regulated by oxidative stress at the level of nuclear localization.

In fission yeast, CRM1 exports certain proteins from the nucleus such as the transcription factor Pap1 [Toone et al., 1998] and the stress activated kinase Sty1/Spc1 [Gaits and Russell, 1999]. Likewise, a mammalian homologue of CRM1, known as Exportin 1 (EXO1), performs a similar function in animal cells. To examine whether CRM1 controls the localization of OXS1, pWS26 was introduced into the *crm1* temperature

sensitive mutant TP17-13C. At 25°C, green fluorescent signals were found in the cytoplasm in most of the cells, as expected if CRM1 is functional in mediating nuclear exclusion. Two hours after raising the temperature to 37°C, the fluorescent signals in nearly all of the cells were found in both the cytoplasm and the nucleus (Figure 2d). This translocation to the nucleus was not detected in wild type cells, despite the elevated temperature.

Consistent with CRM1-mediated nuclear exclusion is the response to leptomycin B (LMB), a *Streptomyces* metabolite that blocks CRM1 interaction with the nuclear export sequence of the cargo protein [Kudo et al., 1998]. After a 1-hour treatment of 50 ng/ml of LMB, the green fluorescent signals in wild type cells harboring pWS26 were detected not only in the cytoplasm, but also in the nucleus. Figure 2e shows a typical pattern taken 2 hours after treatment. The GFP signals were still seen in the nucleus as well as the cytoplasm up to 8 hours after the LMB treatment. The data are consistent with the conclusion that CRM1 exports OXS1 under normal growth, but not during oxidative stress. In the absence of CRM1 function, whether by inactivation by high temperature of a heat sensitive CRM1, or by LMB inactivation of wild type CRM1, OXS1 is detained in the nucleus. The control EGFP-EGFP fusion protein failed to show significant difference with regards to CRM1, or LMB. Deletion analysis of the OXS1 protein reveals a functional nuclear export sequence (NES) in the middle of the polypeptide that shares sequence similarity to the NES of other Exportin/CRM1-regulated proteins [Henderson et al., 2000].

A known response to Cd stress in *S. pombe* is the induced production of phytochelatins, metal binding peptides with the structure of $(Glu\text{-}Cys)_n$-Gly, where the number of Glu-Cys units is typically between 2 to 5. Phytochelatins bind Cd and the metal peptide complex is sequestered into the vacuole by the ABC transporter HMT1. Overexpression of *hmt1*$^{+}$ has been shown to enhance Cd tolerance [Ortiz, 1992], and conversely, the loss of HMT1 function increases Cd sensitivity. When the *oxs1*$^{+}$ cDNA was expressed in the *hmt1*$^{-}$ strain LK100, the transformant was as sensitive to Cd as LK100 harboring an empty vector. Therefore, with respect to Cd tolerance, the *oxs1*$^{+}$-enhanced resistance to this metal in a wild type host cannot operate in the absence of the phytochelatin response.

<u>*Arabidopsis* homologue</u>. Probing with the *Arabidopsis* homologue *At-OXS1* cDNA, the steady-state levels of mRNA were determined from 7 day old *Arabidopsis* seedlings, and from roots, leaves, flowers, and aerial parts from *Arabidopsis* plants just before the bolting stage. A single band hybridizing to the *At-OXS1* cDNA and was present in similar amounts in all tested organs.

At-OXS1 transcript levels were also examined in 7-day old *Arabidopsis* seedlings treated with 30 uM of $CdSO_4$ or 750 uM of diamide. Samples were taken from 0.5, 1, 3, 6, 9 and 24 hours after treatment. A change of *At-OXS1* transcript level was not detected. Hence, like the *S. pombe oxs1*$^{+}$, regulation of *At-OXS1* at the level of transcript accumulation was not observed.

To test whether *At-OXS1* is regulated at the level of protein subcellular distribution, an EGFP-At-OXS1 fusion construct was transformed into *Arabidopsis*. In the absence of stress, the fluorescent signals were observed mainly in the cytoplasm. Upon addition of diamide to the growth medium, or in the absence of diamide but the presence of LMB, the signals were much more visible in the nucleus. Hence, it appears that the *Arabidopsis* OXS1 protein has the same stress inducible relocation properties as does *S. pombe* OXS1, and is most likely a true ortholog of the fission yeast protein.

References

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Fig. 1. Overexpression of *OXS1* genes in *S. pombe*

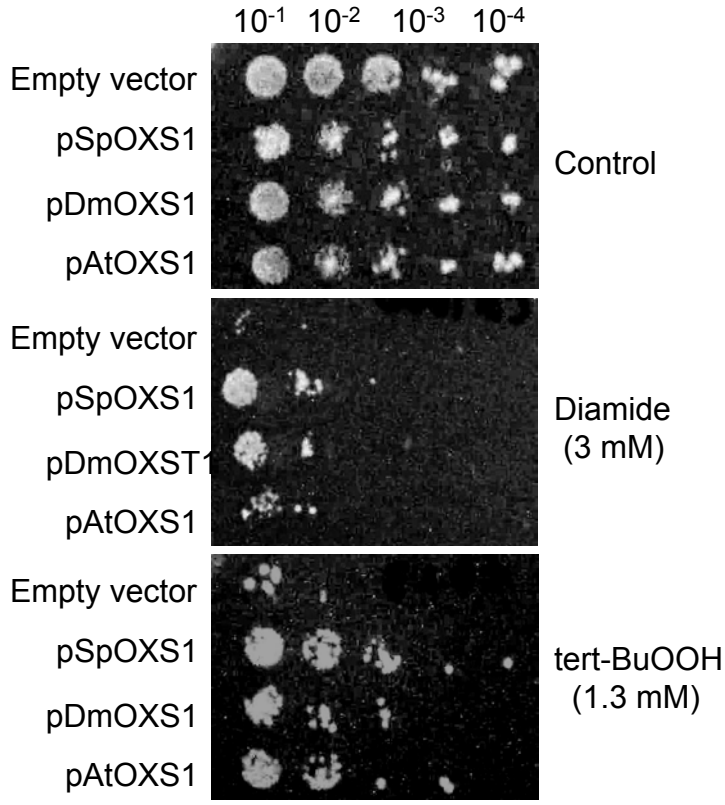
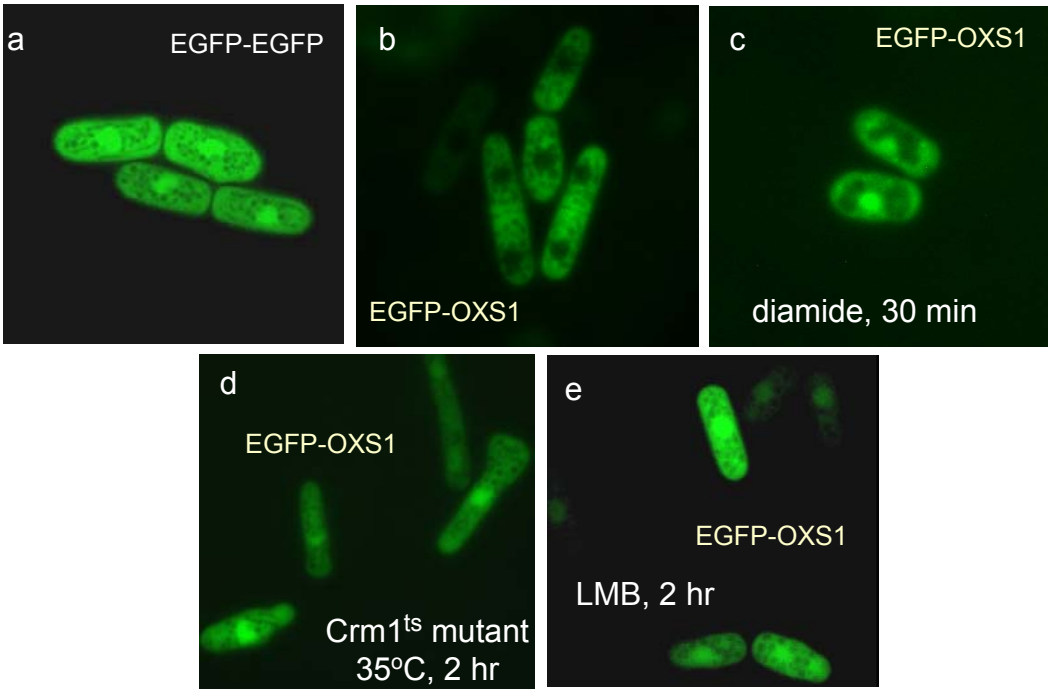
Fig. 2. Cytoplasmic localization of EGFP-OXS1