Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 3867–3871, April 1994
Plant Biology

Redox modulation of the expression of bacterial genes encoding cysteine-rich proteins in plant protoplasts

(thiols/dithiothreitol/neomycin phosphotransferase/β-glucuronidase/thionin)

MANUEL PIÑEIRO, FRANCISCO GARCÍA-OLMEDO, AND ISABEL DIAZ*

Laboratorio de Bioquímica y Biología Molecular, Escuela Tecnica Superior de Ingenieros Agrónomos, Universidad Politecnica Madrid, E-28040 Madrid, Spain

Communicated by Clarence A. Ryan, January 21, 1994

ABSTRACT **Activity of neomycin phosphotransferase II (NPTII; gene, *neo*; five cysteines) in tobacco protoplasts transfected with fusions of the octopine TR2′ or cauliflower mosaic virus 35S promoter and the *neo* gene, with or without a signal peptide, increased up to 8-fold in response to externally added dithiothreitol at concentrations that did not affect protoplast viability (up to 2.5 mM). Activity of phosphinothricin acetyltransferase (PAT; gene, *bar*; one cysteine) expressed under control of the TR1′ or 35S promoter was not similarly affected, thus excluding a redox modulation of transcription as the mechanism of NPTII activation by dithiothreitol. Western-blot analyses showed an increase in the amount of protein in response to dithiothreitol, whereas neither the steady-state level of NPTII mRNA nor the specific activity of the purified enzyme was affected. The same type of modulation was observed for transiently expressed β-glucuronidase (nine cysteines) produced from a fusion with the 35S promoter, with or without a signal peptide. Limitation of cotranslational and/or early posttranslational steps by excessively oxidizing sulfhydryl/disulfide redox potentials is postulated to explain the low net accumulation of cysteine-rich proteins of bacterial origin (i.e., NPTII and β-glucuronidase) when expressed in plant protoplasts, and the marked increase in such proteins in response to externally added dithiothreitol.**

Redox modulation is thought to play a key role in plant metabolism, especially in the regulation of photosynthate formation and utilization (1), and a redox-activated expression of specific plant genes has been proposed (2). We previously showed that a bacterial enzyme, β-glucuronidase (GUS), was inactivated by oxidized thionins, cysteine-rich plant polypeptides, both *in vitro* and when transgenically expressed in plant protoplasts, and that the inactivation seemed to occur more rapidly *in vivo* (3). This differential response suggested the involvement of plant redox mechanisms in the *in vivo* inactivation. Several recent studies carried out with animal cells have shown that externally added reducing agents can perturb disulfide bond formation and interfere with a variety of processes affecting proteins translocated into the endoplasmic reticulum (ER), such as assembly and secretion (4), folding, oligomerization, and intracellular transport (5–7), and sorting among different pathways (8). Dithiothreitol (DTT), a membrane-permeant thiol reducing agent, does not seem to appreciably affect translation, translocation, or early covalent modifications, such as glycosylation or signal-sequence removal, or the function of the secretory pathway and the Golgi complex, whereas it is able to prevent disulfide bond formation in newly synthesized proteins and to induce reduction of partially oxidized folding intermediates in the ER (5, 7).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

We have investigated transient expression in plant protoplasts of a gene encoding a cysteine-rich enzyme of bacterial origin, neomycin phosphotransferase II (NPTII; gene, *neo*, five cysteines), and have found that expression of *neo* is posttranscriptionally modulated by DTT, whether the protein is placed in the cytosol or in the ER. The gene *gus*, encoding another bacterial cysteine-rich enzyme (GUS, nine cysteines), responded to DTT in the same manner as *neo*, while expression of the gene *bar*, encoding phosphinothricin acetyltransferase (PAT), an enzyme with just one cysteine, was not affected. The possible relationship of these results to a lack of adaptation of cysteine-rich bacterial proteins to the more oxidizing sulfhydryl/disulfide (SH/SS) redox potentials in eukaryotic environments is discussed.

MATERIALS AND METHODS

Isolation and Treatment of Protoplasts. Leaf protoplasts were isolated from axenic shoot cultures of transformed [with 35S–*gus* or 35S–*neo* fusions, where 35S represents the cauliflower mosaic virus (CaMV) 35S promoter] and nontransformed *Nicotiana tabacum* W38 by established procedures (9). Purified protoplasts were plated at 2.5–5 × 10^5 per ml in MSP_19M medium (9) and incubated for 20 hr at 27°C in the dark, in the presence of the indicated concentrations of DTT and/or thionins. A mixture of α1 and β thionins from wheat was the gift of A. Molina (Escuela Tecnica Superior de Ingenieros Agrónomos, Madrid). Protoplasts were transiently transfected with the various constructions by electroporation (10) at 48 Ω, 500 μF, 750 V/cm for 20–30 msec with an Electro Cell Manipulator 600 (BTX electroporation system). Protoplast viability was determined by staining with Evan's blue (11).

Plasmids. Plasmid pDE212 contains the coding sequences for NPTII under the control of the TR2′ promoter in the octopine TR promoter fragment (12) and the PAT coding sequence under the control of the TR1′ promoter, which acts divergently with respect to TR2′ out of the same TR DNA piece (12). In pDE210, a gene fragment encoding the signal peptide (SP) of the pathogenesis-related protein 1b replaces the first codon of the *neo* coding region in construction pDE212 (13). Plasmids pDE108 and pDE317 contain the coding sequences for NPTII and PAT, respectively, under the CaMV 35S promoter (13). All pDE constructions were kindly donated by J. Denecke (Uppsala Genetic Center, Ultuna, Sweden). In addition, fusions involving the coding sequence for GUS and the CaMV 35S promoter, with or without the SP from the wheat α-thionin gene (35S–*gus* and 35S–SP–*gus*), were from our previous work (3).

Abbreviations: BSA, bovine serum albumin; CaMV, cauliflower mosaic virus; DTT, dithiothreitol; ER, endoplasmic reticulum; GUS, β-glucuronidase; NPTII, neomycin phosphotransferase II; PAT, phosphinothricin acetyltransferase; SP, signal peptide.

*To whom reprint requests should be addressed.

Enzymatic Assays. To determine NPTII activity, protoplasts were washed twice in MSP_19M medium (9) and extracted in 50 mM Tris·HCl, pH 7.0/0.03% bovine serum albumin (BSA)/2 mM DTT. NPTII activity in this extract and in the culture medium was measured as described (14), except that precipitation with ammonium sulfate was omitted. To avoid any artifactual effect due to the presence of DTT in the culture medium, all samples were brought to 2.5 mM DTT before the reactions were started. For assays involving purified NPTII, test tubes were pretreated with 50 mM Tris·HCl, pH 7.0/0.03% BSA. Purified NPTII was purchased from 5 Prime → 3 Prime, Inc. Radioactivity of [^{32}P]phosphorylated neomycin retained by Whatman P81 paper was counted by excising a 2-cm circle around each spot and transferring it to scintillation fluid (Aquasol, New England Nuclear). GUS activity was measured by fluorometric assay (15). To determine PAT activity, [^{14}C]acetylated phosphinothricin was separated from the reaction mixture by thin-layer chromatography as described by Denecke et al. (13). Each tube was sampled three times at 10-min intervals and the linearly increasing optical density of the autoradiographic images was determined densitometrically. Experiments were repeated at least three times.

RNA Extraction and Hybridization. RNA was extracted by freezing protoplasts in liquid N_2, thawing in extraction buffer [1% (wt/vol) 1,5-naphthalenedisulfonic acid/4% (wt/vol) *p*-aminosalicylic acid], and extracting sequentially with one volume of water-saturated phenol and one volume of chloroform. Nucleic acids were ethanol precipitated, and RNA was selectively precipitated overnight in 3 M LiCl at 4°C. RNA concentration was determined by measuring the absorbance at 260 nm. Northern blots and slot blots were made according to Sambrook *et al.* (16) and hybridized with a 353-bp, ^{32}P-labeled probe obtained from the *neo* coding region by cutting plasmid pDE108 with restriction enzymes *Pst* I and *Sph* I. A ribosomal cDNA probe was used on a duplicate to ascertain that equal amounts of RNA had been loaded.

Protein Electrophoresis and Western Blotting. Cellular extracts and culture media were brought to 70% saturation with ammonium sulfate in the presence of 0.03% BSA. Precipitated proteins were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) in 4–20% polyacrylamide minigels (Bio-Rad). Silver staining of proteins was carried out with the Bio-Rad kit. Proteins were blotted to Immobilon P filters (Millipore) by established procedures. NPTII present in the extracts was visualized by using an anti-NPTII antibody (5 Prime → 3 Prime, Inc.), according to the supplier's instructions.

RESULTS

Increased NPTII *in Vivo* Activity in Response to DTT. To investigate the effect of a more reducing environment on the *in vivo* expression of NPTII, tobacco protoplasts were transfected with gene fusions based on the TR2′ promoter and the coding region of *neo*, with or without a SP sequence (TR2′–*neo*, TR2′–SP–*neo*), and exposed for 20 hr to various concentrations of DTT. A significant increase of NPTII activity in response to DTT was observed (Fig. 1), whether the enzyme was placed in the cytosol (TR2′–*neo*) or translocated into the lumen of the ER (TR2′–SP–*neo*). In the latter case, the protein was exported and DTT did not interfere with export. Western blot analyses of intracellular NPTII, in the TR2′–*neo* experiment, and of extracellular NPTII, in the case of TR2′–SP–*neo*, showed that DTT treatment increased the amount of detectable NPTII protein (Fig. 2). These experiments indicated that the increased activities were the result of marked increases in the net accumulation of the enzymatic

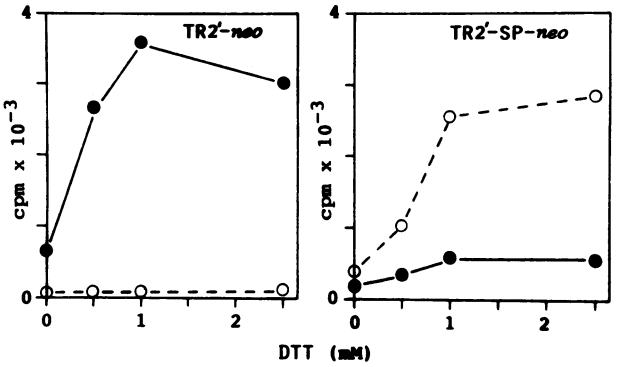

FIG. 1. Effect of DTT on transient expression of chimeric genes TR2′–*neo* and TR2′–SP–*neo* in tobacco protoplasts. Activity corresponding to 1×10^5 and to 5×10^5 protoplasts was measured in each case, respectively. NPTII activity was determined in the cell fraction (●) and in the culture medium (○). No significant activity was detected in mock-electroporated (carrier DNA only) protoplasts which had been incubated in the presence of the various concentrations of DTT.

protein and not of direct or indirect changes in specific activity.

Lack of Activation of Purified NPTII by DTT. To ascertain that DTT itself did not increase specific activity, *in vitro* experiments were carried out which showed that purified NPTII was not activated by DTT, whereas it was inactivated by oxidized thionins (Fig. 3). As previously described for GUS (3), inactivation was accompanied by a decrease in intensity of the NPTII band in the silver-stained electrophoresis gel, which was probably due to the formation of a disulfide-mediated thionin–NPTII complex that was unable to migrate into the gel. Although the reducing agent did not increase enzyme activity, it was able to prevent the inactivation by oxidized thionin, when added simultaneously, and to reverse it when added after 2 hr of incubation with thionin (Fig. 3*B*). Externally added thionin inactivated NPTII in the protoplast, at concentrations that had little or no effect on its viability, as judged by staining with Evan's blue, and the effect was also overcome by DTT (Fig. 4). Activity of NPTII was increased by <10% after a 1-hr exposure to DTT of protoplasts that had been synthesizing the enzyme overnight in the absence of the reagent, and the activity did not change significantly over the same period when DTT was withdrawn from protoplasts that had been incubated overnight in its presence (data not shown). These *in vivo* experiments also indicated either a lack of sensitivity to DTT of the correctly folded functional protein, as previously observed for some animal proteins (7), or that the eventual reduction of disulfide bridges did not alter enzymatic activity, whereas the interaction with an oxidizing agent led to inactivation.

Protoplast viability after isolation and electroporation was not affected by DTT up to 5 mM, even when the reagent was included in the electroporation medium, as judged by staining with Evan's blue after 20 hr (48–52%) and by the proportion

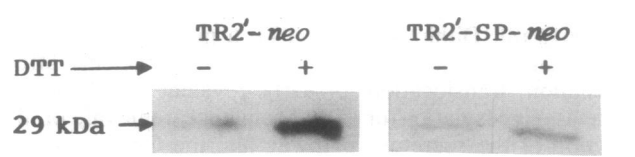

FIG. 2. Western blot analysis of NPTII protein accumulated inside the protoplast, for the TR2′–*neo* construction, and in the medium, for the TR2′–SP–*neo* construction, in the absence (−) or presence (+) of 2.5 mM DTT. Extracts corresponding to 2.5×10^5 and to 1×10^6 protoplasts were loaded for TR2′–*neo* and TR2′–SP–*neo*, respectively. The NPTII band was not detected in mock-electroporation controls (data not shown).

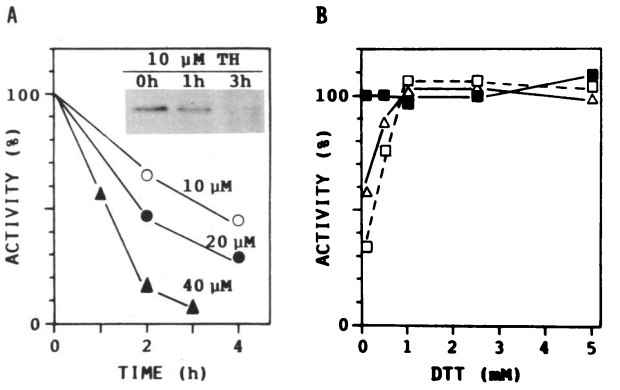

FIG. 3. *In vitro* inhibition of purified NPTII by thionin and its reversal by DTT. (*A*) Time courses of NPTII inhibition by the indicated concentrations of thionin. (*Inset*) NPTII band silver-stained after SDS/PAGE. (*B*) Effect of DTT on the purified enzyme by itself (■); simultaneously mixed with 20 μM thionin and various concentrations of DTT, and incubated for 2 hr (△); and mixed with 20 μM thionin, incubated for 2 hr, and then mixed with various concentrations of DTT and incubated for another 2 hr (□).

of cells undergoing first division after 10 days of culture (43–44%). Both the untreated and the treated protoplasts could be equally regenerated into whole plants. At 10 mM DTT, viability was reduced by 50% and no expression of transgenes was observed. These observations excluded differences in protoplast viability as the factor responsible for the differential net accumulation of protein.

Lack of Effect of DTT on Promoter Activity or on NPTII mRNA Level. Possible promoter involvement in the observed effect was investigated by carrying out transfections with different promoter–gene combinations. In tobacco protoplasts transiently expressing *neo* under control of the 35S promoter, an increase of NPTII activity in response to DTT was observed which was similar to that obtained with the TR2′ promoter (Fig. 5). In contrast, PAT activity was not increased when *bar* was expressed either under the 35S promoter or under the TR1′ promoter (Fig. 5). These results showed that the DTT response did not occur at the transcriptional level and suggested that the effect was specific for cysteine-rich proteins.

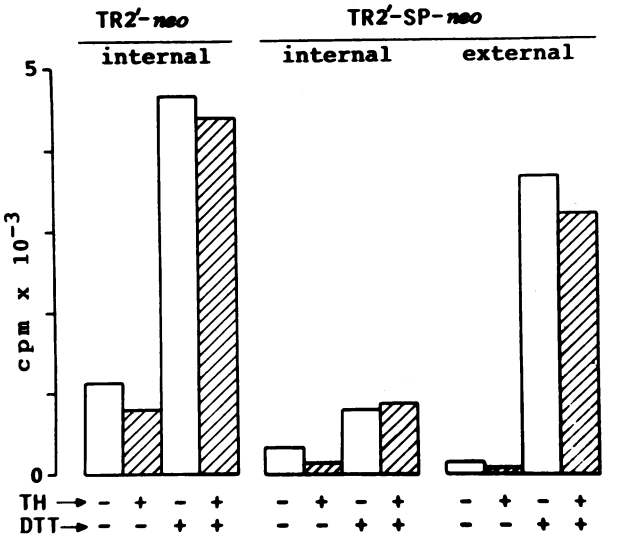

FIG. 4. Inhibition by thionin of transiently expressed NPTII in protoplasts and its prevention by DTT. Protoplasts were incubated for 20 hr in the absence (−) or presence (+) of 20 μM thionin (TH) and/or 1 mM DTT. Inhibition was significant ($P < 0.01$) in the absence of DTT and not significant in its presence.

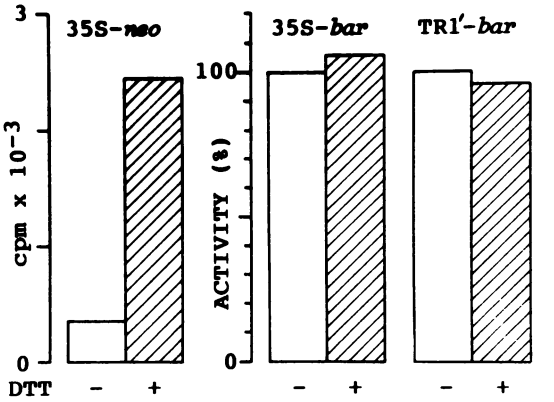

FIG. 5. Effect of DTT on transient expression of *neo* (*Left*) and *bar* under two different promoters (*Right*). The experiments were carried out in the absence (−) or presence (+) of 2.5 mM DTT for 20 hr. NPTII activity was determined as indicated in Fig. 1 and PAT was determined densitometrically; the slope of the product vs. time for the untreated sample was taken as 100% activity.

Protoplasts from tobacco plants transgenically expressing a 35S–*neo* gene fusion increased NPTII activity only by 30–50% in response to a 20-hr exposure to 2.5 mM DTT (data not shown). This suggested that DTT increased the net accumulation of newly synthesized NPTII, whereas it had little effect on the fate of that already existing in the transgenic protoplasts when the treatment was started. The result was in line with the more general observation that DTT did not affect turnover of cysteine-rich proteins during a 24-hr chase of protoplasts that had been pulse-labeled with [^{35}S]cysteine (unpublished data).

Total RNA was extracted from DTT-treated and untreated tobacco protoplasts that had been transfected with the 35S–*neo* construction and incubated for 20 hr. Northern blots of these RNA samples gave single bands with a probe from the coding region of the *neo* gene (data not shown) and slot blot analyses showed that DTT treatment did not alter the steady-state level of NPTII mRNA (Fig. 6).

Together, the above results pointed to cotranslational or early posttranslational events, possibly associated with the status of the cysteines in the nascent or just-completed polypeptide, as those affected by the redox modulation.

Increased GUS Activity by Treatment with DTT. To corroborate the previous idea, expression of GUS, also a cys-

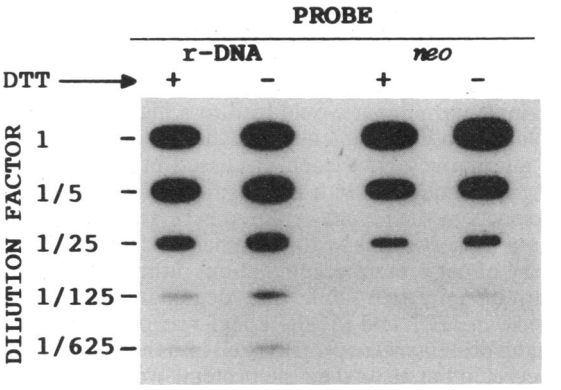

FIG. 6. Slot blot analysis of the effect of DTT on steady-state level of NPTII mRNA. Tobacco protoplasts transfected with the 35S–*neo* gene construction were incubated for 20 hr in the presence (+) or absence (−) of 2.5 mM DTT. Serial dilutions of 2 μg of total RNA from each sample were blotted. One replica was hybridized with a ribosomal cDNA probe (r-DNA) and the other with the probe for the *neo* gene. Exposure for the r-DNA probe (1×10^6 cpm) was 12 hr, and for the *neo* probe (1×10^7 cpm) it was 4 days.

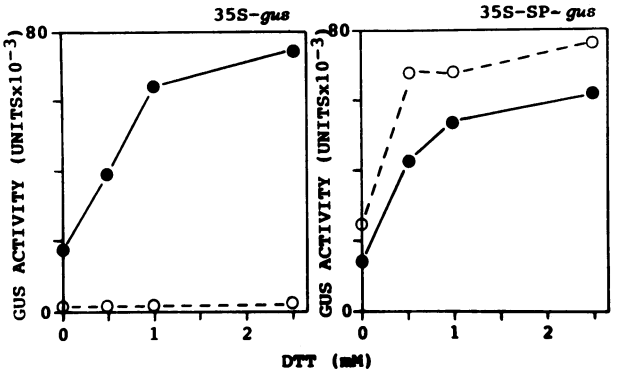

FIG. 7. Effect of DTT on transient expression of chimeric genes 35S–*gus* and 35S–SP–*gus* in tobacco protoplasts. Activity respectively corresponding to 1×10^5 and 2×10^5 protoplasts was determined in each case (1 unit = 1 pmol of 4-methylumbelliferone per min per mg of protein). GUS activity was determined in the cell fraction (●) and in the medium (○).

teine-rich bacterial enzyme, was investigated. GUS activity increased in response to DTT in a manner similar to that of NPTII when the gene fusions 35S–*gus* and 35S–SP–*gus* were transiently expressed in tobacco protoplasts (Fig. 7). In the second case, translocation of GUS into the lumen of the ER resulted in its inactivation by glycosylation, so the experiment was carried out in the presence of tunicamycin (5 μg/ml), which prevented inactivation by blocking glycosylation. The translocated protein was exported, and the export was not affected by DTT. As previously reported (13), the export index (external/internal concentrations) was lower than for NPTII (Figs. 1 and 7). GUS activity in protoplasts from transformed plants expressing the 35S–*gus*/35S–*neo* construction was moderately increased, by about 50%, after treatment with 2.5 mM DTT for 20 hr (data not shown), a response of the same order of magnitude as that observed for NPTII.

DISCUSSION

The above results indicated that the effect of DTT on gene expression was restricted to the cysteine-rich proteins and seemed to occur during or shortly after translation. The lack of effect of DTT on transiently expressed PAT indicated that the efficiency of the translation machinery itself was not affected, in agreement with previous observations with mammalian cells (7). Thus, it was plausible to speculate either that redox changes could affect a rate-limiting step in the folding of the nascent cysteine-rich proteins, which would perhaps determine their overall rate of synthesis, or that the more oxidizing environment would lead to a higher proportion of misfolded proteins, which would be eventually degraded. Both alternatives have been discussed in a recent review, mostly in connection with mammalian cells (17). However, two aspects of the results reported here are in contrast with previous observations with mammalian cells (5, 7): (*i*) an increase of net protein accumulation, rather than a decrease due to incomplete folding and degradation, occurred in response to DTT and (*ii*) the effect was observed not only when the proteins were translocated into the lumen of the ER and secreted but also when the proteins were synthesized in the cytosol, although expression levels were always about 10-fold higher in the latter environment. All these results suggested that the SH/SS redox potentials of both intracellular media in the tobacco protoplast were too oxidizing for maximal expression of NPTII and GUS, two enzymes of bacterial origin. It has been reported that glutathione (GSH) is the principal redox buffer in mammalian cells, and that the GSH/GSSG ratios ranged from approximately 1:1 to 3:1 in the secretory pathway and from 30:1 to 100:1 in the cytosol, whereas typical ratios for *Escherichia coli* are from 50:1 to 200:1 (18). Plant protoplasts grown in the dark, as it is done for transient expression of transfected genes, can be presumed to have a more oxidizing status than illuminated cells (1). Both NPTII and GUS are retained in the cytoplasm of *E. coli*, an environment that is thought to prevent the formation of disulfide bonds (18–20). To the extent that differences in SH/SS redox potentials can explain why *E. coli* cannot properly fold cysteine-rich recombinant proteins of eukaryotic origin (18), it can be inversely argued that the more oxidizing eukaryotic environments could lead to a lower efficiency in the accumulation of properly folded cysteine-rich recombinant proteins of bacterial origin. These environments would tend to favor the production of misfolded proteins, perhaps with spurious disulfide bonds, that would be eventually degraded.

The above observations do not exclude that certain promoters, such as that of cytosolic Cu/Zn superoxide dismutase, are susceptible of redox modulation (2). In the study of the redox induction of that particular gene, GUS was used as the reporter enzyme, but the experiments were carried out with protoplasts from transgenic plants, so that it was likely that only a negligible fraction of the marked increases in GUS activity observed in response to DTT could be ascribed to the phenomenon reported here.

Both stimulation and inhibition of secretion by reducing agents have been reported for various cysteine-containing proteins in mammalian cells (4, 21). Both NPTII and GUS are exported through a default pathway when provided with a SP (13) and the above results indicated that this pathway was not affected by DTT (up to 2.5 mM), although we did observe that an unidentified protein from tobacco protoplasts was retained when treated with this reagent (unpublished data).

Finally, it should be pointed out that genes *neo* and *gus* have been universally used as reporters in transient-expression experiments carried out to analyze a high number of promoters. The observations reported here imply that those experiments were performed at markedly suboptimal conditions for the expression of the reporter genes.

Gift of plasmids by Dr. J. Denecke and critical reading of the manuscript by Drs. P. Carbonero, J. Paz-Ares, and J. Imperial are gratefully acknowledged. Technical assistance was provided by D. Lamoneda and J. García, and financial support was from the Dirección General de Investigación Científica y Técnica (Grant PB92-0325).

1. Cséke, C. & Buchanan, B. B. (1986) *Biochim. Biophys. Acta* **853**, 43–63.
2. Hérouart, D., Van Montagu, M. & Inzé, D. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 3108–3112.
3. Díaz, I., Carmona, M. J. & García-Olmedo, F. (1992) *FEBS Lett.* **296**, 279–282.
4. Alberini, C. M., Bet, P., Milstein, C. & Sitia, R. (1990) *Nature (London)* **347**, 485–487.
5. Braakman, I., Helenius, J. & Helenius, A. (1992) *EMBO J.* **11**, 1717–1722.
6. Braakman, I., Helenius, J. & Helenius, A. (1992) *Nature (London)* **356**, 260–262.
7. Tatu, U., Braakman, I. & Helenius, A. (1993) *EMBO J.* **12**, 2151–2157.
8. Chanat, E., Weiss, U., Huttner, W. B. & Tooze, S. A. (1993) *EMBO J.* **12**, 2159–2168.
9. Power, J. B., Davey, M. R., McLellan, M. & Wilson, D. (1989) *Laboratory Manual Plant of Tissue Culture* (Univ. of Nottingham, Nottingham, U.K.).
10. Saunders, J. A., Smith, C. R. & Kaper, J. M. (1989) *Biotechniques* **7**, 1124–1131.
11. Gaff, D. F. & Okong'O-ogola, O. (1971) *J. Exp. Bot.* **22**, 756–758.
12. Velten, J., Velten, L., Hain, R. & Schell, J. (1984) *EMBO J.* **3**, 2723–2730.

13. Denecke, J., Botterman, J. & Deblaere, R. (1990) *Plant Cell* **2,** 51–59.
14. Denecke, J., Gosselé, V., Botterman, J. & Cornelissen, M. (1989) *Methods Mol. Cell. Biol.* **1,** 19–27.
15. Jefferson, R. A. (1987) *Plant Mol. Biol. Rep.* **5,** 387–405.
16. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
17. Jaenicke, R. (1991) *Biochemistry* **30,** 3147–3161.
18. Hwang, C., Sinskey, A. J. & Lodish, H. F. (1992) *Science* **257,** 1496–1502.
19. Pollit, S. & Zalkin, H. (1983) *J. Bacteriol.* **153,** 27–32.
20. Derman, A. I. & Beckwith, J. (1991) *J. Bacteriol.* **173,** 7719–7722.
21. Hurtley, S. M. & Helenius, A. (1989) *Annu. Rev. Cell Biol.* **5,** 277–307.