Expression of sodCp and sodB genes in Nicotiana tabacum: effects of light and copper excess

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Abstract

Light and copper are necessary for the biogenesis and normal function of chloroplasts, but are also known to initiate the production of active oxygen species. Tobacco (Nicotiana tabacum L.) contains two plastid-located superoxide dismutases, Cu/Zn-SOD and Fe-SOD, encoded by the nuclear genes sodCp and sodB, respectively. The expression of both genes is developmentally regulated and influenced by oxidative stress. Here, the effects of light and copper excess on the expression of these two genes were analysed in seedlings and in leaves of non-flowering tobacco plants. The accumulation of both transcripts was induced by light in seedlings and in young leaves of mature plants, but changes in enzymatic activities were only observed in seedlings. Copper excess was induced in two experimental systems. In the first, axenically-grown plants were transferred to the medium containing excess copper and grown for 10 d. Excess copper induced an increase of sodCp mRNA level, but not of enzymatic activity, whereas Fe-SOD activity and sodB transcript level decreased. In contrast, after transfer of hydroponically grown plants to a copper excess medium and growth for an additional 3 d, sodB mRNA and enzyme levels increased, whereas the sodCp transcript level was reduced. Changes in the expression levels of both genes were more pronounced in young leaves. The effects of light and copper on the expression of plastid-located SODs are discussed.

Key words: Copper excess, light, Nicotiana tabacum L., sodB, sodCp.

Introduction

Three types of superoxide dismutase (SOD, EC 1.1.15.1) have been described in plants: mitochondria-located Mn-SOD, plastid-located Fe-SOD, and Cu/Zn-containing SODs (Bowler et al., 1994; Kanematsu and Asada, 1994). Cu/Zn-SODs are divided into two classes, a cytosolic and a plastidic isoform that are encoded by sodCc and sodCp genes, respectively (Bowler et al., 1994; Kanematsu and Asada, 1994; Zilinskas et al., 1994). SodCp cDNAs have been isolated from a number of plant species (for a review, see Gressel and Galun, 1994), including aspen (Akkapeddi et al., 1994) and tobacco (Kurepa et al., 1997a). The expression of the sodCp gene has been shown to be modulated by a variety of developmental and environmental stimuli (Peri-Treves and Galun, 1991; Strid, 1993; Madamanchi et al., 1994; Wingesle and Karpinski, 1996; Donahue et al., 1997; Kurepa et al., 1997a). The sodB cDNAs, encoding Fe-SOD, were isolated from Nicotiana plumbaginifolia L. and Arabidopsis thaliana L. Heynh. (Van Camp et al., 1990) and from soybean (Crowell and Amasino, 1991a). The expression of sodB has been shown to be developmentally regulated (Crowell and Amasino, 1991b; Casano et al., 1994; Kurepa et al., 1997a) and affected by environmental stimuli (Tsang et al., 1991; Casano et al., 1994; Kurepa et al., 1997a, b).

The effects of light on the expression of genes encoding plastid-located SODs have been described for some plants. Etiolated tomato seedlings contained substantial levels of sodCp transcript (Perl-Treves and Galun, 1991). The steady-state level of this transcript was further augmented by very low fluences of white light. However, in mature plants exposed even to strong fluences, no light-regulated
control of sodCp was detected (Perl-Treves and Galun, 1991). The promoter region of the tomato sodCp gene that contains light-responsive motifs has also been described (Kardish et al., 1994). Light-induced accumulation of sodCp transcript has also been described in spinach (Sakamoto et al., 1993). Tsang et al. (1991) reported that white light induced the accumulation of sodB transcript in dark-adapted mature plants and in etiolated seedlings.

The effects of metal supply on the expression of SODs have been studied in bacteria (for a review, see Fee, 1991), yeast (for a review, see Jamieson, 1995), and many animal species (for a review, see Bannister et al., 1987). In plants, SOD activity and apoprotein levels have been shown to be influenced by the concentration of the active-site metals in the growth medium (del Rio et al., 1991; Chongpraditnun et al., 1992; Iturbe-Ormaetxe et al., 1995; Herbik et al., 1996; Kampfenkel et al., 1995; Kurepa et al., 1997b; Polle et al., 1992). However, in most of the studies it was not investigated whether the observed changes are the result of transcriptional, translational, or post-transcriptional regulation.

Copper (Cu) is a trace element required by all living organisms, but it is also a potent cellular toxin. Cu catalyses the formation of active oxygen species, such as O$_2^-$, H$_2$O$_2$ and OH that are toxic to living systems (Chaudière, 1994; Koppenol, 1994). Because of its necessity and its toxicity, both of which are derived from the ability of the Cu ion to function as an electron transfer intermediate, Cu homeostasis must be tightly regulated. After the entry of an excess of Cu ions into the cell, several protective mechanisms are induced. One line of defence is based on the sequestration by metallothionein-like proteins (Robinson et al., 1993) and phytochelatins (Steffens, 1990). Another line of defence involves low-molecular weight antioxidants (for example, glutathione, ascorbic acid, α-tocopherol, and carotenoids) and antioxidant enzymes, such as SOD. Yeast Cu/Zn-SOD has the characteristics of a Cu-stress protein; the enzyme and its mRNA level increase under conditions of Cu excess (Greco et al., 1990). The ACE1 transcriptional regulator, which is responsible for the induction of yeast metallothionein in response to Cu, also induces the transcription of the Cu/Zn-SOD gene (Carri et al., 1991; Gralla et al., 1991). Chongpraditnun et al. (1992) reported that in Cu$^{2+}$-treated soybean roots cytosolic Cu/Zn-SOD is induced. This increase can be due to either a direct effect of Cu ions on SOD gene transcription or an indirect effect through an increase in levels of superoxide radicals (Chongpraditnun et al., 1992).

Light and Cu are necessary for normal development of plants, but are known to induce and catalyse the formation of oxylipids. The main goal of the present study was to determine whether light and Cu excess treatments of tobacco plants were accompanied by changes in the levels of sodCp and sodB mRNA as well as in the corresponding enzymatic activities. The observed effects, including a lack of correlation between changes in sod mRNA levels and the corresponding enzymatic activities, differential regulation of sodCp and sodB genes, and the age-related loss of the capacity for their induction as well as the involvement of metals in the regulation of sod genes, are discussed.

Materials and methods

Light treatments

Nicotiana tabacum L. cv. Petit Havana SR1 plants were used in all experiments. To evaluate the effects of light on the expression of sodCp and sodB genes, two experimental designs were used. In the first, plants were grown on half-strength Murashige and Skoog medium (MS/2) containing 2.25 g l$^{-1}$ MS salts (ICN Biomedical, Costa Mesa, CA), 3% (v/w) sucrose, 2.5 mM 2-(N-morpholino)ethanesulphonic acid (pH 5.7) and 8 g l$^{-1}$ agar (Difco, Detroit, MI) for 10 d in the dark or light (100 μmol m$^{-2}$ s$^{-1}$). In the second experimental design, plants were grown in soil until the 13-leaf stage in greenhouse conditions at 22°C and 60% relative humidity (with a 16/8 h light/dark regime). Plants were then transferred to a controlled-environment chamber set up at 22°C and 60% relative humidity and kept in the dark for 72 h. After the dark adaptation, plants were exposed to continuous white light (100 μmol m$^{-2}$ s$^{-1}$). Young (the first three apical leaves) and mature (the fourth and the fifth) leaves of two plants were harvested at regular time intervals during dark adaptation and subsequent exposure to light, and frozen for RNA and protein analyses.

Copper treatments

For the hydroponic cultures, seeds were germinated for 10 d on quartz sand moistened with a saturated CaSO$_4$ solution. After transfer to vermiculite, seedlings were supplied with the nutrient solution defined by Kampfenkel et al. (1995). After 4 weeks, plants were transferred to the hydroponic cultures set up in a controlled-environment chamber at 24°C, 60% relative humidity, 130 μmol m$^{-2}$ s$^{-1}$ light intensity in a 16/8 h light/dark regime. The nutrient solution was changed every 10 d and the transpiration and evaporation losses were restored every second day. After 3 weeks of growth in the hydroponic culture, roots were trimmed with a scalpel and Cu$^{2+}$ was added as CuSO$_4$ to a final concentration of 1, 10 and 100 μM. The hydroponic growth was continued for an additional 3 d prior to sampling. For each sample, either two complete plants or only young and mature leaves of two plants were pooled and frozen for RNA and protein analysis.

For plants grown in axenic cultures, seeds were germinated and grown for 2 months on MS/2 media in a controlled-environment chamber at 23°C, 100 μmol m$^{-2}$ s$^{-1}$ light intensity with a 16/8 h light/dark regime. To determine the effects of Cu excess, plants were carefully transferred onto fresh MS/2 media containing either 0.5 μM or 5 μM CuSO$_4$. After 10 d of growth, aerial parts of four plants were pooled for each treatment and used for protein and RNA extraction.

RNA isolation and RNA gel blot analysis

Total RNA was extracted from frozen material as described by Logemann et al. (1987). For RNA gel blot analyses, samples of 12 μg total RNA were fractionated on 1.5% agarose-
Results

Effects of light on expression of sodCp and sodB genes

To test the effects of light, the sodCp and sodB transcript levels and corresponding enzyme activities were examined in etiolated seedlings and in leaves of dark-adapted mature plants. Effects of light on the expression of sodB have been described (Tsang et al., 1991). The sodCp transcript was detected in etiolated seedlings, and its level increased as a result of exposure to light (Fig. 1A). This expression pattern is similar to the one described for the sodB transcript levels in an identical experimental setup (Tsang et al., 1991). The transcript was approximately 5-fold more abundant in light-grown than in etiolated seedlings. When etiolated seedlings were exposed to light for 5 h, the transcript level increased and reached the level detected in light-grown seedlings. A 24 h long exposure of etiolated seedlings to white light resulted in a further increase of sodCp mRNA level. The enzymatic activity of plastid-located SODs in etiolated, light-grown, and etiolated seedlings exposed to light for 1 d is presented on Fig. 1B. The most prominent effect of light was observed for Fe-SOD. The activity of this enzyme was higher in light-grown than in dark-grown seedlings, and it increased after etiolated seedlings were exposed to white light.

In a second experimental set up, mature non-flowering plants were dark-adapted for 3 d and then exposed to continuous white light. Young and mature leaves were collected for RNA and protein extraction at regular time intervals during dark adaptation and subsequent exposure to continuous light. In young leaves, both mRNAs species were found to have a similar trend of accumulation during dark adaptation and subsequent light exposure (Fig. 2). After 24 h of dark, both sodCp and sodB mRNAs levels decreased to approximately 10% of the control. The steady-state level of both transcripts increased upon exposure to white light. Transcript accumulation was not rapid and the transcript levels were similar to those of the control only after approximately 24 h. No changes in enzymatic activities were detected (Fig. 3). In mature leaves, no changes of steady-state level of sodCp were observed (data not shown).

Effects of Cu excess on sodCp and sodB expression in tobacco seedlings

To determine the effects of exposure to excess Cu, 2-month-old axenically-grown plants were transferred to MS/2 media containing 0.5 μM or 5 μM Cu²⁺ and grown for an additional 10 d. No visible signs of toxicity (no
Fig. 2. Effects of dark adaptation and subsequent exposure to light on sodCp and sodB mRNA levels. Young leaves of two soil-grown plants were pooled after the indicated periods and used for total RNA isolation. RNA was separated by a denaturing gel system, blotted onto a membrane, and hybridized to sodCp, sodB, and rRNA probes. After the autoradiography, the hybridization signals were quantified densitometrically. Data were corrected for loading differences using signal intensity obtained with the control rRNA probe and are presented as mean ± SEM (n = 2).

Fig. 3. SOD isozyme profiles in young leaves of plants kept in the dark for the denoted time interval and returned to continuous light. The experimental set up was as for Fig. 2. In this experiment, 20 μg of total protein was loaded per lane. SOD isoforms are denoted on the left-hand-side.

growth retardation, turgor loss, or root necrosis) were observed. The sodCp transcript level was 3-fold and 2-fold higher in plants grown on media containing 0.5 μM and 5 μM Cu2+, respectively (Fig. 4). The sodB mRNA level decreased with increasing Cu concentration to approximately 50% of the control level for 0.5 μM Cu2+ and to 25% for 5 μM Cu2+ treatment (Fig. 4). No changes of chloroplast Cu/Zn-SOD activity levels were detected, whereas Fe-SOD activity was reduced (Fig. 5).

Effects of Cu excess on sodCp and sodB expression in non-flowering tobacco plants

Cu excess in plants grown in hydroponics cultures was induced after trimming the roots and subsequent growth for 3 d in nutrition solution containing 1, 10 or 100 μM CuSO4. The root uptake system was circumvented by partial removal of the roots, which permitted uncontrolled uptake of water-soluble compounds through the transpiration stream in the light (Kampfenkel et al., 1995). After

3 d of growth on 1 μM Cu2+, plants did not show any visual signs of toxicity. Growth on 10 μM Cu2+ was also not retarded. However, young leaves were darker than leaves of control plants and of plants grown on 1 μM Cu2+, whereas fully expanded leaves were chlorotic and wilted (loss of turgor was apparent 24 h after addition of Cu2+). The colour of the roots changed from white to brown and adventitious roots were short and thickened suggesting an inhibition of root elongation. Plants grown for 3 d on 100 μM Cu2+ were stunted and all leaves were wilted and chlorotic with necrotic spots at the tips proceeding along the leaf blades.

Analyses of the RNA isolated from the whole plants demonstrated that while the 1 μM Cu2+ treatment did not influence the sodCp mRNA level, growth on 10 and 100 μM Cu2+ resulted in a reduction to approximately 20% of the control level (Fig. 6A). The sodB transcript...
SodCp and sodB expression

Fig. 6. Effects of Cu excess on sodCp and sodB mRNA accumulation and Fe-SOD and chloroplast Cu/Zn-SOD abundance in hydroponically grown plants. (A) Non-flowering, 8-week-old tobacco plants were transferred to nutrient solution containing 1, 10 or 100 μM Cu²⁺ and grown for 3 d. Aerial parts and roots of two plants were pooled and used for total RNA isolation. RNA gel blot analyses and the quantification of the data were the same as for Fig. 2. (B) Total protein extracts were separated on an IEF gel (pH 4–6.5), preincubated in CN⁻-containing buffer and stained for SOD activity. (C) Total protein extracts (40 μg per well) were separated on 15% SDS/PAGE, blotted and probed with anti-Fe-SOD antibodies. Molecular mass of prestained SDS/PAGE standards is indicated (Bio-Rad, Hercules, CA).

The level was approximately 3-fold higher in plants grown on 1 μM Cu²⁺, whereas growth on 10 and 100 μM Cu²⁺ resulted in its 2-fold increase compared to that of control (Fig. 6A). Changes in enzymatic activities have been reproducibly detected only for Fe-SOD (Fig. 6B; data not shown). Analyses of IEF gels stained for SOD activity after preincubation with CN⁻, which inhibits the activity of Cu/Zn-SOD isoforms, showed that Fe-SOD activity increased after Cu treatment (Fig. 6B). To confirm this result, immunoblotting analyses with anti-Fe-SOD antibodies were performed. The increase of Fe-SOD activity in plants grown on toxic concentrations of Cu²⁺ was due to an increase in the level of the Fe-SOD apoprotein (Fig. 6C).

To determine whether Cu treatments differentially affect the expression of sodCp and sodB in leaves of different age, young and mature leaves of two plants were pooled and used for protein and RNA extractions. RNA gel blot analyses showed that the sodCp mRNA level was 2-fold higher in young leaves of plants grown on 1 μM Cu²⁺, whereas in plants grown on 10 μM Cu²⁺ the level was 50% that of the control (Fig. 7). The Cu²⁺ treatments did not significantly affect the steady-state level of sodCp mRNA in mature leaves. The sodB mRNA level was 4-fold higher in young leaves of plants grown on 1 μM Cu²⁺, with the level remaining 3-fold higher in plants grown on 10 μM Cu²⁺. As for sodCp, the sodB mRNA levels in mature leaves did not significantly change after the treatment. Immunoblotting analysis showed that the increase in sodB levels in young leaves was accompanied by an increase in Fe-SOD apoprotein, whereas no changes of Fe-SOD apoprotein levels were detected in mature leaves (Fig. 8).
Discussion

Lack of correlation between sod mRNA and enzyme levels

The expression of both sodCp and sodB genes seems to be light regulated in seedlings as well as in mature plants. At this stage, it is difficult to conclude whether the observed responses reflect a direct effect of light on gene expression or are due to an excess of active oxygen species produced by illumination. Changes in enzymatic activity could be detected only in seedlings. This lack of correlation between the sod transcript levels and enzymatic activities of SOD isoforms is a recurring theme (Williamson and Scandalios, 1992; Karpinski et al., 1992, 1993; Madamanchi et al., 1994; Wingsle and Karpinski, 1996; Kurepa et al., 1997a, b). It has been suggested that increased oxidative stress leads to an increased production of signals that induce the transcription of sod genes. On the other hand, increased stress would result in a higher turnover of SODs also through an increase of their inactivation by the end-product of the dismutation reaction, H$_2$O$_2$ (Scandalios, 1993; Karpinski et al., 1993).

Thus, as the end result of oxidative stress, higher transcript levels but unchanged enzymatic activities are detected. However, cases where reduction of sod mRNA levels is accompanied by constant (Fig. 3) or even higher level of SOD apoprotein or enzyme (Madamanchi et al., 1994) have also been described. Reduction of sod mRNA levels, followed by a reduction in enzyme activities has been reported as well (Kurepa et al., 1997b). Therefore, one can speculate that the SOD enzyme levels reflect not only transcriptional but also post-transcriptional regulation of sod genes.

Differential regulation of sod genes suggests distinct functions

The cellular toxicity of Cu is primarily a result of its ability to catalyse the formation of active oxygen species through a series of redox reactions (Koppenol, 1994). In plants grown in hydroponic cultures on 10 and 100 μM Cu$^{2+}$, visual signs of Cu toxicity were observed and levels of sodCp transcript were reduced whereas sodB mRNA levels increased. This is a second frequently observed phenomenon: as a result of oxidative stress sodCp transcript levels are reduced (Strid, 1993; Madamanchi et al., 1994; Donahue et al., 1997; Kurepa et al., 1997a), whereas sodB transcript levels are increased (Tsang et al., 1991; Kurepa et al. 1997a). Therefore, chloroplast Cu/Zn-SOD and Fe-SOD catalyse the same chemical reaction but are functionally different. The specific functions of these two enzymes are still speculative. Fe-SOD has been suggested to protect chloroplasts from superoxide radicals produced by the photosynthetic electron transport chain (Tsang et al., 1991). Chloroplast Cu/Zn-SOD has been associated with the protection from superoxide radicals produced during dark metabolism or chloroplast biogenesis (Slooten et al., 1995).

Age-related loss of capacity and/or sensitivity for sod induction

It has been shown that total SOD activity in young oat leaves was progressively higher with increasing concentrations of Cu$^{2+}$ up to 10 mM, whereas in older leaves the SOD activity remained constant above 100 μM Cu$^{2+}$ (Luna et al., 1994). The stress-induced expression of sod genes in barley was also modulated by the developmental stage of the organ (Casano et al., 1994). Sod genes seem to become gradually less sensitive for induction by photodamagingise with increasing leaf age (Casano et al., 1994). Here, no pronounced changes in sodCp and sodB mRNA levels have been detected in mature leaves after light and Cu excess treatments. This observation would suggest that a loss of capacity for sod induction during senescence is a common phenomenon in all plants and might apply to a variety of stressors.

Metal-regulated transcriptional control of sod genes

This study shows that prolonged growth on media containing non-toxic concentrations of Cu salts resulted in the accumulation of sodCp mRNA. In contrast, under these conditions sodB mRNA and Fe-SOD activity levels decreased. Thus, mRNA profiles are opposite from the ones described for plants grown hydroponically (Figs 4, 6A). Judging from the observed phenotypes, the Cu doses used for the seedling experiment were not toxic. Additionally, the root uptake system was circumvented in plants grown in hydroponic cultures. Thus, one can assume that the changes in sod mRNA levels in seedlings grown for longer time periods on Cu-supplemented media reflect an adaptation response. It is therefore tempting to speculate that the observed increase in sodCp mRNA level is a result of a Cu-mediated transcriptional control rather than of an induction by oxidative stress.

Uptake of excess Cu can induce deficiency of other essential elements and may dramatically affect the cation balance in the cell (Welch, 1995). In intact roots, an inverse correlation has been found between Fe availability and Cu accumulation (Robinson et al., 1993). In pea leaves, measurements of Fe and Cu content have shown that the level of Cu indeed increased after growth on Cu excess media, whereas the Fe content was significantly reduced (Palma et al., 1987). The transcription of the sodB has been reported to be regulated by Fe availability (Kurepa et al., 1997b). Thus, the reduction of the sodB mRNA level in plants grown on media with excess Cu, which therefore might have lower iron content, possibly reflects the Fe-mediated component of sodB transcriptional control.

Phylogenetic distribution of Fe-SOD and chloroplast
Cu/Zn-SOD in plants is still a matter of debate (Bowler et al., 1994). In a majority of cases, Cu/Zn-SOD is the only detectable chloroplastic isoform. Fe-SOD activity has been detected only in a number of unrelated plant families (Bowler et al., 1994). A number of theories have been proposed to explain the apparently random occurrence of Fe-SOD (Bridges and Salin, 1981). The most plausible theory is that the sodB gene exists in all plant species, but is not expressed constitutively. If environmental determinants can lead to preferential expression of one of the plastid-located SODs (Bowler et al., 1994), then availability of SOD metal cofactors in the growth media may be an important factor. The data presented here and the results of Barón Ayala and Sandmann (1988), who showed that Fe-SOD was only detected in Cu-depleted pea leaves, would support this hypothesis. Thus, the availability of Fe, Cu and Zn in the growth medium, as well as the species-specific differences in the mechanisms that regulate the final cellular content of these metals, could lead to the preferred expression of either chloroplast Cu/Zn-SOD or Fe-SOD.

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