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Research Paper

# Thioredoxin (Trxo1) interacts with proliferating cell nuclear antigen (PCNA) and its overexpression affects the growth of tobacco cell culture

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ABSTRACT

Thioredoxins (Trxs), key components of cellular redox regulation, act by controlling the redox status of many target proteins, and have been shown to play an essential role in cell survival and growth. The presence of a Trx system in the nucleus has received little attention in plants, and the nuclear targets of plant Trxs have not been conclusively identified. Thus, very little is known about the function of Trxs in this cellular compartment. Previously, we studied the intracellular localization of PsTrxo1 and confirmed its presence in mitochondria and, interestingly, in the nucleus under standard growth conditions. In investigating the nuclear function of PsTrxo1 we identified proliferating cellular nuclear antigen (PCNA) as a PsTrxo1 target by means of affinity chromatography techniques using purified nuclei from pea leaves. Such protein–protein interaction was corroborated by dot-blot and bimolecular fluorescence complementation (BiFC) assays, which showed that both proteins interact in the nucleus. Moreover, PsTrxo1 showed disulfide reductase activity on previously oxidized recombinant PCNA protein. In parallel, we studied the effects of PsTrxo1 overexpression on Tobacco Bright Yellow-2 (TBY-2) cell cultures. Microscopy and flow-cytometry analysis showed that PsTrxo1 overexpression increases the rate of cell proliferation in the transformed lines, with a higher percentage of the S phase of the cell cycle at the beginning of the cell culture (days 1 and 3) and at the G2/M phase after longer times of culture (day 9), coinciding with an upregulation of PCNA protein. Furthermore, in PsTrxo1 overexpressed cells there is a decrease in the total cellular glutathione content but maintained nuclear GSH accumulation, especially at the end of the culture, which is accompanied by a higher mitotic index, unlike non-overexpressing cells. These results suggest that Trxo1 is involved in the cell cycle progression of TBY-2 cultures, possibly through its link with cellular PCNA and glutathione.

## 1. Introduction

In plants, abiotic and biotic stresses usually interfere with the redox state of the cells, leading to the generation of excess reactive oxygen and nitrogen species (ROS/RNS) that affect plant growth and development under normal and stress conditions. In addition, ROS and RNS are known to act as signaling molecules in the maintenance of physiological functions and in the response to changing environments [1–3]. In green tissues, although the main sources of ROS are chloroplasts and peroxisomes, mitochondria account for the total production, generating ROS as a product of respiration [4,5], while several reports point to the nuclear compartment as being particularly sensitive to the deleterious effects of oxidation [6]. Furthermore, recent evidence shows that ROS and, particularly $H_2O_2$, generation occurs as a consequence of DNA damage, suggesting that the nuclear generation of oxidants may also function in cell signaling. In plant cells, experiments

*Abbreviations:* AMS, 4-acetamido-4-maleimidylstilbene-2,2-disulfonic acid; BiFC, bimolecular fluorescence complementation; BSA, bovine serum albumin; DTT, 1,4-dithiothreitol; DAPI, 4,6-diamidine-2-phenylindol; DEM, diethyl maleate; GFP, green fluorescent protein; mCBM, monochlorobimane; NTR, NADPH thioredoxin reductase; OD, optical density; Oex, overexpressing; PCNA, proliferating cell nuclear antigen; Prx, peroxiredoxin; RNA, reactive nitrogen species; ROS, reactive oxygen species; RT-qPCR, Reverse transcription quantitative polymerase chain reaction; TBS, Tris-buffered saline; TFs, transcription factors; Trx, thioredoxin; TBY-2, tobacco bright yellow-2; YFP, yellow fluorescent protein

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performed in tobacco (*Nicotiana tabacum* L.) Bright-Yellow-2 (TBY-2) nuclei also suggest that this compartment may not only be invaded by ROS diffusing from neighboring compartments but is also an active source of ROS, in particular of $H_2O_2$ [7], which has been implicated in the regulation of plant development, the cell cycle and the induction of plant defense responses during stress adaptation, as well as in plant cell death [8,9].

These reactive molecules may perturb cellular homeostasis, and some protein cysteine residues are highly sensitive to oxidation due to the reactivity of their thiol groups, which may interrupt cellular homeostasis [10,11]. The redox state of plant thiols and the regulation of cysteinyl residues in proteins are emerging as key players in the response of plants to different stresses, as well as in plant development, functioning in the redox sensing and signal transduction pathways. Thiol reduction is mainly controlled by the thioredoxin (Trx)/peroxiredoxin (Prx) and glutathione (GSH) systems, which modulate redox signaling during development and stress adaptation [12–15]. Thioredoxins are small proteins containing two cysteines in the redox active center and they are involved in the reduction of disulfide bonds of other proteins through a dithiol-disulfide exchange mechanism. In plants there are at least ten families of Trxs, with more than 40 members present in almost all cellular compartments [16]. The diversity of isoforms seems to support the idea that plants have an additional antioxidant system compared to mammals, where only two types of Trxs have been described, Trx1 and Trx2, in the cytosol/nucleus and mitochondria, respectively [17].

The presence of Trx in plant mitochondria was demonstrated in Arabidopsis [18], where it was classified as Trx*o* type (AtTrx*o*1). More recently, we described how pea Trx*o*1 is located in both the mitochondria and nucleus under physiological non-stressed conditions [19], while several cytosolic Trx*h* isoforms accumulate in the nucleus of developing wheat aleurone and scutellum cells during oxidative stress [20], subsequent studies describing a functional thioredoxin system in the plant nucleus [21]. However, in general, little has been published on the presence of Trxs in the nucleus in plants. In mitochondria, and in cytosol, these oxidoreductases are reduced with electrons from NADPH by compartment-specific NADPH/TRX reductases (NTRs), whereas the chloroplast Trxs are reduced by the electrons provided by photosynthetic electron transport [22]. In animal systems, on the other hand, cytosolic/nuclear Trx1 has been well characterized. Stress-induced Trx1 accumulates in the nucleus to get the required redox state of the transcription factors (TFs) to bind the promoter region of DNA, acting as a master regulator of transcription. For example, Trx1 induces transcriptional activity of NF-kB increasing its ability to bind to DNA [23]. It is also required to resist apoptosis, probably by regulating the apoptotic gene p53 [24]. In plants, some redox-regulated TFs under retrograde regulation have been described in response to stress. Trx*h*5 has been reported as involved in the reported redox regulation of NPR1 allowing its translocation to the nucleus, to activate redox-sensitive TGA [25]. In sugarcane, Trx*h*1 has been identified as an interacting partner of the redox-regulated TF SsNAC23, which is a member of the plant-specific NAC TF family, with roles in development and the response to cold stress [26].

Furthermore, previous evidence supports the view that cellular redox homeostasis is a crucial regulator of cell fate in mammals and plants, and that an intrinsic redox cycle consisting of reductive and oxidative phases can exert a major influence over cell cycle progression [27]. Trx has been shown to be necessary for cell-cycle progression in *E. coli* [28] and *Xenopus* [29]: more specifically, its lack in yeast induces cell-cycle arrest at G1 to S phase [30], acting as the physiological electron donor for ribonucleotide reductase (RNR) during DNA precursor synthesis [31,32]. In the human system, Trx is reported to enhance cell growth [33], whereas in plants, the Trx system and glutathione are involved in the control of the postembryonic development of the shoot apical meristems [34]. Moreover, accumulated evidence shows that in both mammalian and plant cells, glutathione (GSH) concentrates in the nucleus in the early phases of cell growth, where it fulfills a number of important functions [35,36].

Recently we reported that the overexpression of PsTrx*o*1 caused significant differences in the response of a TBY-2 cell culture to high concentrations of $H_2O_2$, consisting of a higher and maintained viability in over-expressing cells, while non-overexpressing lines suffered a severe decrease in viability and marked oxidative stress, with generalized and rapid cell death. All these data pointed to PsTrx*o*1 as a pivotal factor responsible for the delay in the programmed cell death provoked by the $H_2O_2$ treatment [37]. However, to the best of our knowledge, there is no information on the functional involvement of Trx*o*1 in the nucleus. Previous biochemical and proteomic approaches have been developed to identify target proteins of different thioredoxins, and among the high number of proteins detected, some were nuclear proteins. Only a few of these candidate proteins have been experimentally validated [38]. Thus, to increase our knowledge of the potential nuclear functions of Trx*o*1, we first followed a proteomic approach to identify possible target proteins, using a purified nuclear preparation obtained from pea (*Pisum sativum* L.) leaves. As a result, proliferating cell nuclear antigen (PCNA) was identified as a putative PsTrx*o*1 target. This was confirmed by dot blot analysis and bimolecular fluorescence complementation (BiFC) assays, which revealed that PsTrx*o*1 and PCNA interact in the nucleus. PCNA is a key component of the DNA replication machinery present in the nuclei of all dividing cells, where it plays a central role connecting different DNA metabolic pathways [39,40]. Therefore, to further understand the relationship between both Trx*o*1 and PCNA proteins, an *in vitro* oxido-reductase enzymatic assay was carried out. Furthermore, PsTrx*o*1 overexpressing TBY-2 cell cultures were used to study the function of thioredoxin in living cells, analyzing the effects on cell growth as well as on the PCNA and glutathione content and its cellular localization by flow cytometry. Taken as a whole, the flow cytometry results suggest that Trx*o*1 is involved in cell cycle progression, possibly providing a reductive nuclear environment and interacting with PCNA. All these data may represent a key aspect linking the influence of Trx*o*1 on GSH and PCNA to the observed changes in TBY-2 cell cycle progression.

## 2. Material and methods

### 2.1. Plant material, culture growth conditions and growth measurement

Stable PsTrx*o*1 over-expression lines of *Nicotiana tabacum* 'Bright Yellow-2′ (TBY-2) suspension cells were generated as reported [37], and two of these lines and a control GFP line were used. The suspension of tobacco cells was routinely propagated and cultured at 26 °C and a stationary culture was diluted 4:100 (v/v) in new medium according to [41]. The growth of the cell culture was measured by optical density at 600 nm [42].

### 2.2. Protein extracts

50 mL of TBY-2 culture at different days of growth were centrifuged at 3000*g* for 5 min at 4ºC and resuspended in extraction buffer 100 mM Tris-HCl pH 7.5, 20 mM DTT, 10 mM EDTA, 0.2% Triton X-100, 1 mM PMSF. After 30 1-s-long pulses of sonication on ice, the homogenate was centrifuged at 15,000*g* for 15 min at 4ºC and the supernatant was incubated with 1% streptomycin sulfate for 20 min at room temperature to precipitate the DNA after centrifugation at 15,000*g* at room temperature. The supernatant was kept at 80 °C until use for western blot analysis. Total proteins were determined using the Bradford assay [43].

### 2.3. Antibodies and recombinant proteins

Monoclonal antibody against PCNA (clone PC10, mouse) was