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Characterization of the *Arabidopsis thaliana* 2-Cys peroxiredoxin interactome

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ABSTRACT

Peroxiredoxins are ubiquitous thiol-dependent peroxidases for which chaperone and signaling roles have been reported in various types of organisms in recent years. In plants, the peroxidase function of the two typical plastidial 2-Cys peroxiredoxins (2-Cys PRX A and B) has been highlighted while the other functions, particularly in ROS-dependent signaling pathways, are still elusive notably due to the lack of knowledge of interacting partners. Using an *ex vivo* approach based on co-immunoprecipitation of leaf extracts from *Arabidopsis thaliana* wild-type and mutant plants lacking *2-Cys PRX* expression followed by mass spectrometry-based proteomics, 158 proteins were found associated with 2-Cys PRXs. Already known partners like thioredoxin-related electron donors (Chloroplastic Drought-induced Stress Protein of 32 kDa, Atypical Cysteine Histidine-rich Thioredoxin 2) and enzymes involved in chlorophyll synthesis (Protochlorophyllide OxidoReductase B) or carbon metabolism (Fructose-1,6-BisPhosphatase) were identified, validating the relevance of the approach. Bioinformatic and bibliographic analyses allowed the functional classification of the identified proteins and revealed that more than 40% are localized in plastids. The possible roles of plant 2-Cys PRXs in redox signaling pathways are discussed in relation with the functions of the potential partners notably those involved in redox homeostasis, carbon and amino acid metabolisms as well as chlorophyll biosynthesis.

1. Introduction

Peroxiredoxins (PRXs) are a family of non-heme peroxidases able to reduce H_2O_2 and organic peroxides using thiols as electron donors [1]. Their catalytic activity is carried out by a conserved peroxidatic cysteine (Cys^P). Typical 2-Cys PRXs are active as a homodimer and possess a second conserved resolving Cys (Cys^R) [2]. The 2-Cys PRX catalytic cycle consists of Cys^P oxidation by peroxide, generation of a sulfenic acid form (Cys^P-SOH), formation of a disulfide bond with Cys^R and reduction of this bond by an electron donor related to the thioredoxin (TRX) family [3]. Upon pro-oxidative conditions, the sulfenic acid form in 2-Cys PRXs from eukaryotes can be overoxidized to sulfinic (Cys^P-SO_2H) or sulfonic (Cys^P-SO_3H) acid forms leading to inactivation of the peroxidase activity [4]. In *Saccharomyces cerevisiae* and human cells, oxidative treatment or heat shock lead to 2-Cys PRX overoxidation concomitant with modifications in conformation and formation of high molecular weight complexes. This structural modification is linked to a functional switch from peroxidase to chaperone activity [5,6].

Further, in yeast and animal cells, a signaling role of 2-Cys PRXs has been recently highlighted. 2-Cys PRXs can interact with other proteins and regulate their activity as a function of their redox state as shown in yeast for the Tpx1 PRX and the Pap1 transcription factor [7]. Currently, 18 proteins regulated by PRXs have been identified using mainly targeted approaches like co-immunoprecipitation, pull-down or yeast two-hybrid assays. These

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partner proteins are involved in various processes related to activation of stress-responses and phosphorylation signaling pathways, or regulation of cellular differentiation and apoptosis (for review see [8]). Conversely, modulation of the 2-Cys PRX peroxidase activity by binding proteins has also been shown. For example, the peroxidase activity is inhibited by phosphorylation of the Thr^{89} residue in human PRX by the Cdk5-p35 kinase [9] or by interaction with the macrophage migration inhibitory factor (MIF) [10] while the interaction with cyclophilin A increases the peroxidase activity of another type of PRX in mammals [11].

In plants, typical 2-Cys PRXs have been first discovered in barley and spinach [12] and further characterized in *Arabidopsis thaliana*, where two plastidial isoforms (A and B) sharing 85% homology are present. These abundant proteins represent *ca.* 1% of the chloroplastic proteins [13,14]. An *Arabidopsis thaliana* double mutant fully knocked-out for the expression of *2-Cys PRX A* and *2-Cys PRX B* genes has been recently characterized. This mutant displays reduced growth under long day conditions and is more sensitive than wild type (Wt) to high light [15]. It was proposed that 2-Cys PRXs take part in an alternative water-water cycle able to detoxify H_2O_2, protecting the photosynthetic structures against oxidative damage upon environmental constraints [15]. Accordingly, overexpression of 2-Cys PRX in potato plants leads to tolerance against methyl viologen or high temperature [16].

Compared to other organisms, the chaperone and signaling functions of 2-Cys PRXs remain poorly characterized in plants. In Chinese cabbage seedlings, 2-Cys PRX complexes from 60 to 200 kDa are mostly present upon optimal growth conditions while upon stress conditions high molecular-weight complexes (*ca.* 700 kDa) are observed [17]. Separation of these complexes by size exclusion chromatography revealed a peroxidase activity for the former and a chaperone activity for the latter [17]. However, we recently reported no obvious relationship between 2-Cys PRX overoxidation and oligomerization upon physiological environmental constraints [18], suggesting that the 2-Cys PRX chaperone function is not essential *in planta*. So far, few plant 2-Cys PRX partners have been identified [19,20]. Affinity chromatography and co-immunoprecipitation experiments showed that the unusual CDSP32 (Chloroplastic Drought-induced Stress Protein of 32 kDa) TRX reduces and interacts with 2-Cys PRXs [21,22]. Another TRX-related protein, NTRC (NADPH-dependent Thioredoxin Reductase C), efficiently reduces *in vitro* 2-Cys PRXs [20,23]. FRET experiments confirmed this interaction *in vivo* in Arabidopsis protoplasts [24]. In other respects, Dangoor et al. [25] showed that 2-Cys PRXs oxidize an Atypical Cysteine Histidine rich Thioredoxin, ACHT1, and transmit a redox signal regulating the photosynthetic electron transport chain during the day/night transition. Moreover, the ADP-glucose pyrophosphorylase (AGPase) activity is also controlled via the oxidation of another ACHT-type TRX, ACHT4, by 2-Cys PRXs [26]. Finally, plant 2-Cys PRXs have been reported to interact *in vitro* with some proteins which do not belong to the TRX superfamily: an enzyme involved in carbon metabolism, Fructose-1,6-BisPhosphatase (FBPase) [27], an enzyme involved in chlorophyll synthesis, (Protochlorophyllide OxidoReductase B, POR B) [28] and a cyclophilin, Cyp20-3, participating in protein folding [24,29].

The analysis of 2-Cys PRX oligomerization status revealed the presence of the protein in complexes of various sizes in plant extracts [18]. We wondered whether these oligomers could be hetero-complexes formed with partner proteins as reported in other organisms [8]. To test this hypothesis, we developed a non-targeted approach based on co-immunoprecipitation from leaf extracts of *Arabidopsis thaliana* combined with mass spectrometry-based proteomics. This approach led to the identification of numerous proteins potentially associated with plant 2-Cys PRXs.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana (cv. Col-0) plants were grown from sowing in soil under an 8-h photoperiod and a photon flux density of 200 μmol photons m^{-2} s^{-1} with a temperature regime of 22 °C/18 °C (day/night) and a relative humidity of 55% during six weeks. Plants were alternatively watered with tap water and a Coïc-Lesaint nutritive solution [30] every two days. One T-DNA double mutant line for the *2-Cys PRX A* and *2-Cys PRX B* genes, here abbreviated *2cysprx* produced from crossing the GK_295C05 and SALK_017213 lines was used [15,18].

2.2. Protein preparation and co-immunoprecipitation assays

Following leaf grinding in liquid nitrogen, soluble proteins were extracted under native conditions using phosphate buffer pH 7,4 (137 mM NaCl, 47 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) containing 1 mM PMSF, but no reductant to preserve the redox status of protein complexes. Following vigorous shaking at 4 °C for 20 min and centrifugation (20 min, 21,500*g*, 4 °C), the supernatant was stored in ice and immediately used for co-immunoprecipitation. Protein concentration was determined using the "Protein Quantification BCA Assay" kit (Interchim). Co-immunoprecipitation experiments were performed using the Pierce® Co-Immunoprecipitation kit (Ref. 26149, Thermo Scientific) according to manufacturer's recommendations. Antibodies raised against 2-Cys PRX (50 μL of crude serum [21]) were immobilized on 50 μL of resin beads under slow agitation at room temperature for 3 h 30 min. One mg of proteins from crude leaf extracts in 400 μL phosphate buffer was incubated with the resin under slow agitation at 4 °C for 30 min. Flow-through was then collected by centrifugation (1 min, 1,000*g*) and the resin was washed five times before elution using the appropriate buffers. The eluted proteins were separated by SDS-PAGE for either silver nitrate staining, western blot or mass spectrometry analyses.

2.3. Silver nitrate staining

Silver nitrate staining was performed using the method developed by Heukeshoven and Dernick [31]. Briefly, after SDS-PAGE migration, the gel was rinsed three times in distilled water for 5 min and incubated to fix proteins in 50% ethanol and 10% acetic acid for at least 30 min, then incubated in 40% ethanol, 0.8 M sodium acetate, 0.025% (v/v) glutaraldehyde and 8 μM sodium thiosulfate for 30 min. After 3 washings in distilled water, proteins were stained by incubating gels in 6 μM silver nitrate and 0.04% formaldehyde for 30 min, followed by incubation for a few min in 0.24 M sodium carbonate and 0.04% (v/v) formaldehyde. The reaction was stopped in 43 mM Na_2-EDTA and gels were conserved in water to take photographs.

2.4. Immunoblot analysis

Proteins separated in SDS-PAGE gels were electro-blotted onto 0.45 μm nitrocellulose (Pall Corporation) to perform immunoblot analysis. The At2-Cys PRX antiserum raised against the recombinant purified 2-Cys PRX A [21] was used at a dilution of 1:10,000. Bound antibodies were detected using an anti-rabbit immunoglobulin G coupled to alkaline phosphatase (Sigma) at a dilution of 1:10,000.