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

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Highlights :

- The signaling functions of plant 2-Cys peroxiredoxins remain elusive.
- 2-Cys PRX partners have been isolated using a non-targeted approach.
- More than 65 potential plastidial partners have been identified in leaf extracts.
- The approach is validated by the presence of known 2-Cys PRX partners.
- The data provide perspectives for characterizing plant 2-Cys PRX functions.

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.

Keywords: *Arabidopsis thaliana*; co-immunoprecipitation; peroxiredoxin; protein interaction; proteomics.

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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 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

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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.

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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)

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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.

2.5.Mass spectrometry analysis, protein identification and validation

2.5.1. Protein digestion.

Proteins from eluates were stacked in the top of a SDS-PAGE gel (NuPAGE 4-12%, Invitrogen) before Coomassie blue staining (R250, Bio-Rad). Gel bands corresponding were manually excised and cut in pieces before being washed by 6 successive incubations of 15 min in 25 mM NH_4HCO_3 containing 50% (v/v) acetonitrile. Gel pieces were then dehydrated in 100 % acetonitrile and incubated at 53°C with 10 mM DTT in 25 mM NH_4HCO_3 for 45 min and in the dark with 55 mM iodoacétamide in 25 mM NH_4HCO_3 for 35 min. Alkylation was stopped by adding 10 mM DTT in 25 mM NH_4HCO_3 and mixing for 10 min. Gel pieces were then washed again by incubation in 25 mM NH_4HCO_3 before dehydration with 100% acetonitrile. Modified trypsin (Promega, sequencing grade) in 25 mM NH_4HCO_3 was added to the dehydrated gel pieces for an overnight incubation at 37°C. Peptides were then extracted from gel pieces in three 15-min sequential extraction steps in 30 µL of 50% acetonitrile, 30 µL of 5% formic acid and finally 30 µL of 100% acetonitrile. The pooled supernatants were then vacuum-dried.

2.5.2. Nano-LC-MS/MS analyses.

The dried extracted peptides were resuspended in 5% acetonitrile and 0.1% trifluoroacetic acid and analysed by online nanoLC-MS/MS (Ultimate 3000, Dionex and LTQ-Orbitrap Velos pro, Thermo Fischer Scientific). Peptides were sampled on a 300 µm x 5

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mm PepMap C18 precolumn and separated on a 75 µm x 250 mm C18 column (PepMap, Dionex). The nanoLC method consisted in a 120-min gradient ranging from 5% to 45% acetronitrile in 0.1% formic acid at a flow rate of 300 $nL.min^{-1}$. MS and MS/MS data were acquired using Xcalibur (Thermo Fischer Scientific). Spray voltage and heated capillary were set at 1.4 kV and 200°C, respectively. Survey full-scan MS spectra (m/z = 400–1600) were acquired in the Orbitrap with a resolution of 60,000 after accumulation of 10^6 ions (maximum filling time: 500 ms). The twenty most intense ions from the preview survey scan delivered by the Orbitrap were fragmented by collision induced dissociation (collision energy 35%) in the LTQ after accumulation of 10^4 ions (maximum filling time: 100 ms).

2.5.3. Data analyses.

Data were processed automatically using Mascot Daemon software (version 2.5.1, Matrix Science). Concomitant searches against Uniprot (*Arabidopsis thaliana* taxonomy, January 2016 version) and classical contaminant protein sequence (homemade) databases and the corresponding reversed databases were performed using Mascot (version 2.5). ESI-TRAP was chosen as the instrument, trypsin/P as the enzyme and 2 missed cleavage allowed. Precursor and fragment mass error tolerances were set at 10 ppm and 0.6 Da, respectively. Peptide modifications allowed during the search were: carbamidomethyl (C, fixes) acetyl (N-ter, variable) and oxidation (M, variable). The Proline software was used to filter the results (conservation of only rank 1 peptides, peptide identification FDR < 1% as calculated on peptide scores by employing the reverse database strategy, minimum peptide score of 25, peptide length ≥ 7, and minimum of 1 specific peptide per identified protein group) before performing a compilation, grouping and comparison of the protein groups from the samples. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [32] partner repository with the dataset identifier PXD003923.

To be considered as a potential binding partner of 2-Cys PRX, a protein must be identified only in the Wt sample with a minimum specific spectral count of 3 or be found enriched at least 5 times in this sample compared to the *2cysprx* control.

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3. Results and Discussion

3.1. Isolation of 2-Cys PRX partners

To identify putative partners of Arabidopsis typical 2-Cys PRXs, we developed a non-targeted strategy based on co-immunoprecipitation. This *ex vivo* approach has proven to be valuable to isolate physiological partners of various types of plant proteins [22, 33, 34]. Particularly, the use of extracts from non-modified plants provides several advantages including the use of the endogenous non-tagged protein to trap partners. Further, we also used crude leaf protein extracts from *2cysprx* mutant plants as a negative control. The comparison of the silver nitrate profiles and western blot analyses showed the absence of 2-Cys PRX in elution samples from *2cysprx* while it was highly abundant in Wt samples, demonstrating the suitability of the technique to isolate potential 2-Cys PRX partners (Fig. 1). Most interestingly, single bands and stained areas were clearly and specifically detected in elution fractions from Wt samples compared to those from *2cysprx* samples.

Mass spectrometry-based proteomic analysis of the elution fractions recovered from Wt and *2cysprx* plants and spectral counting-based comparison (Suppl. Table 1) led to the identification of 158 proteins specifically present or highly enriched in Wt extracts compared to *2cysprx* extracts (Fig.2, Suppl. Table 2). As expected, 2-Cys PRXs A and B were only found in Wt samples and were the most abundant proteins identified, consistent with the results shown in Fig. 1 and the fact that 2-Cys PRXs form dimers and homo-oligomers [3]. These results are in agreement with our recent report revealing that plant 2-Cys PRXs are present in oligomers with a size range from 40 to 160 kDa, which could be homo-oligomers or hetero-complexes formed with partner proteins [18]. Altogether, these data validate the usefulness of co-immunoprecipitation as a non-targeted approach to identify partners of PRXs. To our knowledge, such a strategy has not been used to trap PRX partners in extracts of native proteins in the plant field, and only once in other organisms to isolate PRX V partners in mouse kidney extracts, allowing the identification of 17 potential partners [35].

3.2. Analysis of identified partners

The 158 proteins found associated to 2-Cys PRXs are involved in various biological functions (Fig. 2 and Suppl Table 2). The main represented groups are related to antioxidant mechanisms, metabolisms of carbon and amino acid, and proteolysis. In this work, we chose

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to use crude leaf extracts for co-immunoprecipitation assays, and not purified chloroplasts where 2-Cys PRXs are localized. Indeed, the method used here to prepare protein samples is rather simple and lasts *ca*. 40 min whereas the purification of chloroplasts on Percoll gradients requires much more time and several centrifugation and resuspension steps, during which destabilization of PRX-partner complexes might occur. However, as a consequence, numerous proteins found to interact with 2-Cys PRX (58%) localize outside plastids (Fig. 3). These interactions can be considered to be non-specific and non-relevant from a physiological point of view, but it is tempting to speculate that non-plastidial partners might constitute partners of the PRXs present in other subcellular compartments like cytosolic PRXs II and nuclear 1-Cys PRX. Among these non-plastidial proteins, we found 15 proteins involved in proteolytic mechanisms, including several subunits of the 26S proteasome. In animal cells, the interaction between the typical 2-Cys PRX 1 and the Omi/HtrA2 protease leads to increased activity of the latter [36]. In other respects, it has been suggested in mouse that the circadian oscillations of 2-Cys PRXs overoxidation are linked to increased degradation by the 20S proteasome [37]. Our results suggest that plant PRXs could also be part of proteolytic complexes and function in processing steps necessary for recognition and/or degradation by the proteasome.

Then, we focused our attention on the partners localized in plastids. We noticed that more than 40% (67) of the 158 proteins identified are localized in this compartment (Fig. 3). Table 1 summarizes the names, numbers of peptides and specific spectral counts obtained for these putative partners. Most interestingly, among these 67 proteins, we identified several previously characterized 2-Cys PRX partners like CDSP32, fructose-1,6-bisphosphatase and PORB [20, 21, 22, 24, 27, 28] or proteins for which gene expression is strongly co-regulated at the transcript level with that of the *2-Cys PRX A* gene like the 50S ribosomal protein L21, and PRX Q [24]. These data confirm the relevance of the co-immunoprecipitation method used to identify 2-Cys PRX partners in plant leaf extracts.

3.2.1. 2-Cys PRX reductants

Among the eleven proteins participating in redox homeostasis and found as potential partners of 2-Cys PRXs, one protein of the TRX family, CDSP32, known as a 2-Cys PRX electron donor is present. CDSP32 is an atypical plastidial TRX displaying an increased abundance upon environmental constraints [38]. Using CDSP32 active-site mutants and affinity chromatography, Broin et al. [21] reported that the TRX forms a hetero-complex with

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the 2-Cys PRX. Moreover, co-immunoprecipitation using antibodies raised against CDSP32 also revealed the CDSP32-2-Cys PRX interaction in potato leaf extracts [22]. Finally, the *in vivo* 2-Cys PRX redox status has been shown to depend on the presence of CDSP32 using various methods [18, 39]. Another atypical TRX, ACHT2, is 5-fold more abundant in Wt than in *2cysprx* samples. Interestingly, this TRX belongs to a family, two members of which, ACHT1 and ACHT4, have been recently identified as plant 2-Cys PRX partners [25, 26]. Taken collectively, these data indicate that CDSP32 and ACHTs likely constitute physiological electron donors to 2-Cys PRXs.

Other TRXs and TRX-like proteins (TRXs y2, x, m1, m2, m4, f1, f2, z, NTRC and h3) have been identified in our study, but display abundances below the fixed cut-offs to decree a protein as a potential binding partner of 2-Cys PRXs (Supp. Table 1). Of note, most of them, except TRX h3, are localized in plastids suggesting that 2-Cys PRXs preferentially bind the TRXs located in this organelle and not the numerous TRX isoforms located in other cell compartments. The interaction between 2-Cys PRXs and NTRC, which has been described as an efficient physiological reductant [18, 40] has been reported based on FRET experiments in Arabidopsis protoplasts [24]. The low enrichment of NTRC in Wt samples in our study could result from the fact that the NTRC-2-Cys PRX complex is formed by homodimers of each component [20] and could be destabilized during the co-immunoprecipitation experimental conditions. In other respects, TRX x, which has been shown as the most efficient 2-Cys PRX electron donor *in vitro* [19], was found in Wt samples below the fixed cut-offs (Suppl. Table 1). In line with this finding, Pulido et al. [41] suggested that this protein was not an essential electron donor *in vivo* for 2-Cys PRXs and Cerveau et al. [18] did not observe any change in the 2-Cys PRXs overoxidation level in an Arabidopsis *trx x* mutant, but substantial modifications in this level in Arabidopsis and potato plants deficient in NTRC or CDSP32, respectively. On the basis of all these data, we can thus presume that typical TRXs are less efficient to reduce plant 2-Cys PRXs than TRX-related proteins such as CDSP32, ACHTs and NTRC.

Finally, SRX, which catalyzes *in planta* the reduction of overoxidized 2-Cys PRXs [42], has not been identified in our study. Site-directed mutagenesis experiments on human SRX and 2-Cys PRXs revealed a direct interaction between the two proteins [43]. The absence of this partner in our work might be explained by a weak interaction between the two proteins or by a very low level of *SRX* expression in plants. Indeed, western blot analysis of Arabidopsis SRX needs a large amount of leaf proteins and leads to a very weak signal [18], likely indicating a very low abundance of the protein. This is in agreement with the available

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data in Arabidopsis on the expression level of *SRX* which is much lower than that of *2-Cys PRX* genes (Arabidopsis eFP Browser; http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).

3.2.2. Other proteins involved in the redox homeostasis maintenance

Regarding the other partners exhibiting antioxidant functions or involved in the maintenance of cell redox homeostasis (Table 1), two proteins implicated in the chloroplastic ascorbate-glutathione cycle have been identified specifically in Wt samples: one glutathione S-transferase (GST), dehydroascorbate reductase 3 (DHAR3), and one glutathione reductase, GR2. This cycle is involved in the removing of the high H_2O_2 amounts produced in chloroplast during the light period [44]. Glutathione reductases are enzymes catalyzing the reduction of oxidized glutathione GSSG into reduced glutathione GSH at the expense of NADPH. GR2 is localized in plastids and is also essential for root growth [45]. DHAR3 is a monomeric GST-like protein which is active as a thiol transferase, but does not exhibit any glutathione conjugating activity like other GSTs [46]. In *Arabidopsis thaliana*, the GST superfamily consists of 53 genes, representing at least six classes [47]. Another plastidial GST, GST F8 has been identified as interacting with 2-Cys PRXs (Table 1). This GST is a marker of early stress/defense responses [48, 49] and belongs to the phi class, which exhibits glutathione conjugating and glutathione peroxidase activities [49]. It is worth mentioning that in human cells, the 1-Cys PRX monomeric type can form complexes with a very distinct type of glutathione-S-transferase (π) sharing *ca.* 20% homology with GSTF8 and DHAR3 [50]. Interestingly, we also found among the partner proteins involved in the glutathione metabolism the first enzyme catalyzing the synthesis of this compound, glutamate-cysteine ligase (GCS) (Table 1). Altogether, these data suggest the occurrence of interplays between 2-Cys PRXs, synthesis of glutathione and detoxification mechanisms depending on this antioxidant compound.

More surprisingly, among the proteins identified as associated to 2-Cys PRXs, we found two isoforms of an enzyme also implicated in the detoxification of ROS, but not related to thiol reductases: the plastidial [Fe] and [Cu-Zn] superoxide dismutases (Table 1), which dismute superoxide ($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2) [51] and are essential in the maintenance of plastidial redox homeostasis, due to the production of superoxide at the level of photosystem I. Of note, [Cu-Zn] SOD, which possesses three cysteines is also a TRX partner [52] and thus could be regulated by various types of redox signals. Moreover, another type of PRX, PRXQ, one of the four PRXs located in plastids and having antioxidant

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functions [53], was also found in the Wt sample. These findings suggest the existence of interactions between the two types of PRXs or the existence of super-complexes containing both types of PRXs and their electron donors. Of note, both PRXs can be reduced by the atypical CDSP32 TRX [22].

The identification of eleven proteins interacting with Arabidopsis 2-Cys PRXs, possessing antioxidant functions and involved in the maintenance of redox homeostasis is consistent with the role of these peroxidases in the control of plastidial redox homeostasis which is a key parameter for the proper development and growth of plants [15, 54]. This suggests that 2-Cys PRXs likely act in concert with enzymatic actors related to the ascorbate-glutathione cycle or enzymes like SODs that detoxify reactive oxygen species (ROS) other than hydrogen peroxide.

3.2.3. Proteins involved in the metabolism of amino acids

Among the eight proteins involved in the metabolism of amino acids (Table 1), we found two members of the 5'-adenylylsulfate reductase (APR) family. APR catalyzes the reduction of activated sulfate to sulfite, a key reaction in the sulfate reduction pathway leading to the synthesis of cysteine and methionine [55, 56]. In addition, it possesses a TRX-like C-terminal domain [55, 57], which could interact with 2-Cys PRXs. This could lead to a thiol exchange between the two types of proteins and regulation of the activity of APR enzymes. We also found ferredoxin-dependent glutamate synthase 1 (Fd-GOGAT) in this group. In *Arabidopsis thaliana*, there are two ferredoxin- or NADH-dependent GOGAT participating in the assimilation of ammonium into glutamate in leaves and roots, respectively [58]. Finally, LL-diaminopimelate aminotransferase, which is involved in the biosynthesis of lysine in plants by catalyzing the interconversion of the tetrahydrodipicolinate and the LL-diaminopimelate [59], has been also detected only in Wt samples. In *Arabidopsis thaliana*, this protein activates defense signaling mechanisms involving salicylic acid [60]. All these data reveal that 2-Cys PRXs are able to interact with enzymes participating in the synthesis of various types of amino acids. Of note, the *Arabidopsis thaliana* mutant knockout for the expression of both *2-Cys PRX A* and *B* genes displays under high light a decreased content in aromatic amino acids [15].

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3.2.4. Proteins involved in protein folding

Our interactomics approach allowed identifying six 2-Cys PRX partners involved in protein folding (Table 1). Two of them, CYP38 and CYP37, display a peptidyl-prolyl cis-trans isomerase activity and belong to the cyclophilin (CYP) family. Interestingly, based on DNA-protecting assays, plastidial 2-Cys PRXs A and B have been reported to interact with another member of the CYP family, CYP 20-3. They have been first proposed to be reduced by the two cysteines of this partner [29]. In our approach CYP 20-3 was found under the fixed cut-off, but 4-fold more abundant in Wt than in *2cysprx* samples (Suppl. Table 1). Muthuramalingam et al. [24] reported that CYP20-3 could promote the dissociation of 2-Cys PRX oligomers *in vitro* and consequently inhibit the chaperone function of these complexes. Recently, it has been proposed that this dissociation is pH-dependent and that 2-Cys PRX–CYP20-3 complexes form a redox-dependent regulatory module in the chloroplast [61]. Most interestingly, it has been also shown that the addition of stromal extracts modulates *in vitro* the dissociation dynamic of these complexes [61]. This indicates that other proteins participate in the stability of these complexes and we can speculate that some 2-Cys PRX interacting proteins identified in this work fulfil such a function. In other respects, the study of *cyp 20-3* mutants in Arabidopsis revealed that CYP20-3 functions in the repair of photodamaged photosystem II and in responses to oxidative stress [62, 63]. Consistently, another potential partner of 2-Cys PRXs, CYP38, is involved in photosystem II assembly by guiding the proper folding of the D1 protein [64]. These data indicate that 2-Cys PRXs, via interactions with CYP proteins, might participate in the maintenance of the photosynthetic structures under environmental constraints.

3.2.5. Proteins involved in chlorophyll synthesis

Four proteins involved in chlorophyll synthesis have been identified (Table 1). In this class, we found a known 2-Cys PRX partner, protochlorophyllide reductase B (POR B). Enzymatic photoreduction of protochlorophyllide to chlorophyllide is the first step in chlorophyll biogenesis and is mediated by POR oxidoreductases [28]. Arabidopsis mutants deficient in NTRC, a described electron donor to plant 2-Cys PRXs, show impaired chlorophyll biosynthesis and accumulate intermediate products preceding the protochlorophyllide synthesis step [28]. Based on *in vitro* assays showing that protochlorophyllide synthesis is enhanced by the addition of the 2-Cys PRX/NTRC system,

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Stenbaeck et al. [28] proposed that this system is important for scavenging H_2O_2 in etiolated plants and protects the machinery involved in chlorophyll biosynthesis. Two glutamate-1-semialdehyde 2.1-aminomutases (GSAs) have been also isolated as 2-Cys PRX partners. These proteins are involved in the synthesis of 5-aminolevulinic acid (ALA), which is the universal precursor of tetrapyrrols, like chlorophyll and heme. The possible interaction of 2-Cys PRXs with enzymes participating in chlorophyll synthesis is consistent with the lower leaf chlorophyll content measured in the *2cysprx* mutant [15] and with the hypothesis of Richter and Grimm [65] who proposed that oxido-reduction mechanisms dependent on NTRC and 2-Cys PRXs modify the activity, folding and stability of these enzymes.

3.2.6. Proteins involved in carbon metabolism

Five plastidial proteins interacting with 2-Cys PRXs are involved in carbon metabolism (Table 1). Among them, we found a known 2-Cys PRX partner in plants: fructose-1,6-bisphosphatase (FBPase). In pea, FBPase has been described as a key enzyme for CO_2 assimilation and coordination of the carbon and nitrogen metabolisms [66]. In other respects, the native and recombinant forms of rapeseed 2-Cys PRX are able to enhance the FBPase activity through a mechanism distinct of that mediated by TRXs and requiring the formation of a disulfide bond between two Cys residues of FBPase, a third Cys residue, the presence of fructose-1.6-bisphosphate and Ca^{2+} [27]. The phosphoglucomutase (PGM) enzyme, only detected in Wt samples, catalyzes the reversible interconversion of glucose-1-phosphate and glucose-6-phosphate [67]. Plastidial PGM is essential for the synthesis of starch in leaves during the day and for its subsequent degradation [68, 69]. Finally, we also isolated three components of the plastidial pyruvate dehydrogenase complex (PDC): pyruvate dehydrogenase E1 subunits beta-2 and alpha 3 and dihydrolipoyllysine-residue acetyltransferase component 5. This complex catalyzes the irreversible oxidative decarboxylation of pyruvate to produce Acetyl-CoA, CO_2 and NADH [70, 71]. 2-Cys PRXs might thus be able to interact with key enzymes of carbon metabolism, which provide the energy required for proper growth. This is consistent with the reduced growth of the Arabidopsis *2cysprx* mutant, even in the absence of environmental constraints [15].

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3.2.7. Other proteins

Ten poorly or not characterized proteins are also present in the list of partners (Table 1). Recent data obtained for some of them provide information about their functions. It is notably the case of quinone oxidoreductase known under the name of alkenal/one oxidoreductase (AOR), an enzyme catalyzing the NADPH-dependent reduction of reactive carbonyls. The elimination of these carbonyls generated due to lipid peroxidation is essential for maintaining the cell redox homeostasis [72, 73]. The identification of this protein is consistent with the presumed initial participation of 2-Cys PRXs in the detoxification of lipid hydroperoxides [74] and the maintenance of redox homeostasis as shown above (cf. 3.2.2.). In this category, we also found two RubisCO accumulation factors (RAF1 and 2), which are chaperone proteins involved in the RubisCO biogenesis. *RAF1* and *RBCL* co-expression in tobacco cells leads to an increase in photosynthesis and growth [75]. These data suggest that 2-Cys PRXs could promote RubisCO folding and carbon assimilation by interacting with RAFs.

4. Conclusions and Perspectives

In the last years, the physiological roles of 2-Cys PRXs in plants have been the subject of much research. The recent production of a double mutant completely devoid of 2-Cys PRXs confirmed the crucial role of these proteins in the maintenance of cell redox homeostasis and most particularly in the regulation of plastidial H_2O_2 concentration [15]. In yeast and mammals, a 2-Cys PRX chaperone role has also been proposed in relation with its level of overoxidation and oligomerization [5, 6]. But recent data gained on plants subjected to physiological environmental constraints showed no substantial and related modifications regarding the amounts of overoxidized and oligomerized protein [18]. Of note, in other organisms, PRXs have been described as sensors/transmitters of redox signals to partner proteins. Thus, in mammals, PRX1 and 2 regulate the activity of 18 proteins as a function of their redox status [8]. Based on these data and on the present work, we propose that plant 2-Cys PRXs besides their peroxidase function could fulfil important roles in various signaling pathways via interaction with partner proteins (Fig. 4).

In plants, a large number, more than 500, of partners of TRXs, the 2-Cys PRXs electron donors, has been identified using *in vitro* approaches mainly based on either labelling of thiol groups followed by electrophoresis or on affinity chromatography using mutated

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TRXs [52]. All these partners contain at least one Cys residue, like most of the 2-Cys PRX interacting proteins identified here. This could mean that 2-Cys PRXs interact with these partners to reduce sulfenic acid Cys forms. Most interestingly, 19 potential targets of 2-Cys PRXs identified in our work have also been reported as known or putative partners of plant TRXs. This is notably the case of proteins involved in antioxidant mechanisms ([Cu/Zn] SOD 2, PRXQ [22, 76, 77]), chlorophyll synthesis (GSA 1 [78]), carbon metabolism (fructose-1,6-bisphosphatase, pyruvate dehydrogenase, phosphoglucomutase [79, 80]), amino acid metabolism (APRs, argininosuccinate synthase [81]) and ferredoxin-related enzymes (GOGAT, NiR and SiR [82-84]) (Table 1). This prompts us to speculate that 2-Cys PRXs form super-complexes including TRXs and common partners, like fructose-1.6-bisphosphatase, which is activated both by TRXs and 2-Cys PRXs through distinct mechanisms [27, 80]. In other respects, it is interesting to note that five proteins getting their reducing power from ferredoxin (Fd-GOGAT, SiR, FNR 1, NiR and ISPG) might interact with 2-Cys PRX. This opens the possibility of complex relationships and interplays between ferredoxin-dependent pathways, since the reduction of 2-Cys PRXs is partly catalyzed by plastidial TRXs, which receive electrons from the ferredoxin-TRX reductase complex (FTR) [85].

The high number of proteins interacting with 2-Cys PRXs indicates that these peroxidases could fulfill functions in sensing and/or transmitting redox signals in plants as reported in animal cells [8]. Plant 2-Cys PRXs contain a conserved threonine residue sensitive to phosphorylation and known to regulate the enzyme activity in mammals [86]. Of note, a phosphorylated form of the rice 2-Cys PRX has been isolated in response to heat shock [87]. But, among the putative 2-Cys PRX partners isolated here, only one kinase (adenosine kinase 1, AK1), non-localized in plastids and non-acting on peptides, has been identified (Suppl. Table 1). The absence of plastidial protein kinases in our data could be due to the weak abundance of these signaling proteins or to a very labile link between kinases and 2-Cys PRXs. The occurrence of interplays between redox-signaling pathways involving 2-Cys PRXs and transduction pathways such as phosphorylation remains thus to be investigated in plants using other strategies than co-immunoprecipitation.

The development of a strategy based on co-immunoprecipitation allowed identifying many potential partners of plant 2-Cys PRXs involved in various key cellular pathways notably related to redox homeostasis, chlorophyll synthesis, amino acids and carbon metabolisms. This is fully consistent with the phenotype of the *2cysprx* mutant, which is

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strongly impaired regarding these processes [15]. Moreover, we found several already well-known plant 2-Cys PRXs partners validating the relevance of this non-targeted approach and giving credence to the physiological significance of our findings. For all other possible partner proteins, for which no relationship has been reported up to now, it will be necessary to determine whether this interaction with 2-Cys PRXs is direct and to validate it *in vivo* using other approaches such as yeast two-hybrid experiments or bimolecular fluorescence complementation. To conclude, co-immunoprecipitation seems a valuable strategy to search partners of 2-Cys PRXs and could be applied in other physiological contexts, for instance non-photosynthetic tissues, like flowers, which also display a relatively high abundance of these peroxidases [18].

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Figure legends

Figure 1: SDS-PAGE and Western analyses of the various fractions collected following co-immunoprecipitation of leaf extracts from *Arabidopsis thaliana* Wt and *2cysprx* plants using 2-Cys PRX antibodies.

Co-immunoprecipitation experiments were performed using soluble leaf proteins from Wt and ***2cysprx*** plants extracted in native conditions. Samples were separated by SDS-PAGE electrophoresis (10 µL per lane) for silver nitrate staining (**A**) or Western blot analysis (**B**) using antibodies raised against Arabidopsis 2-Cys PRXs. The arrows indicate protein bands specifically detected in Wt elution fractions.

L : ladder, F : flow-through, W : washing, E : elution.

Figure 2: Functions of the 158 proteins interacting with 2-Cys PRXs identified by co-immunoprecipitation of leaf extracts of *Arabidopsis thaliana* plants using 2-Cys PRX antibodies and mass spectrometry.

Figure 3: Subcellular localisation of the 158 proteins interacting with 2-Cys PRXs identified by co-immunoprecipitation of leaf extracts of *Arabidopsis thaliana* plants using 2-Cys PRX antibodies and mass spectrometry

Figure 4: A model for plant 2-Cys PRXs functions in various plastidial metabolic processes (based on [15], [18] and this work).

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Figure 1

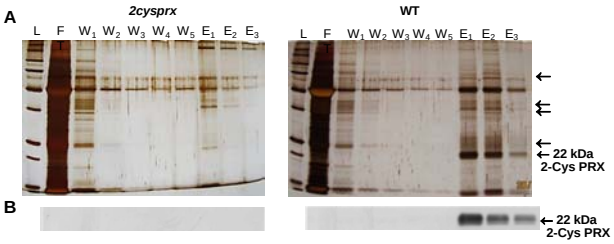

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Figure 2

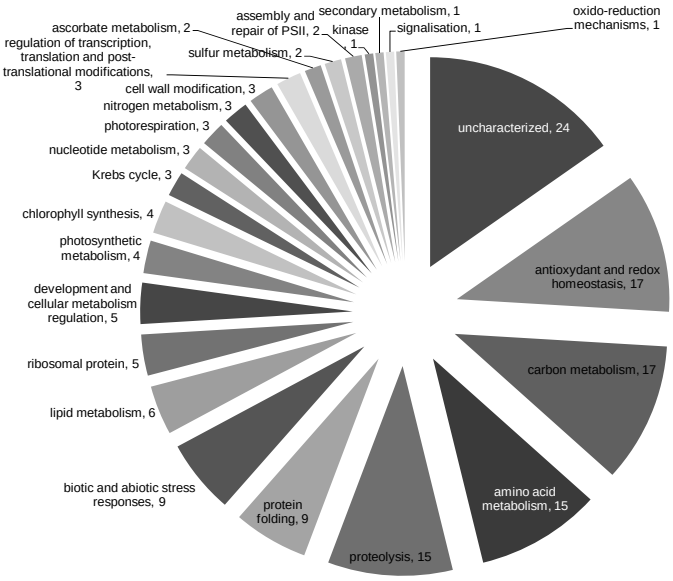

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Figure 3

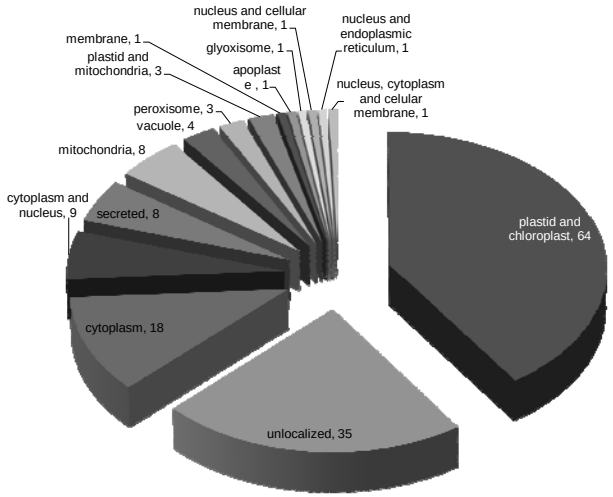

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Figure 4

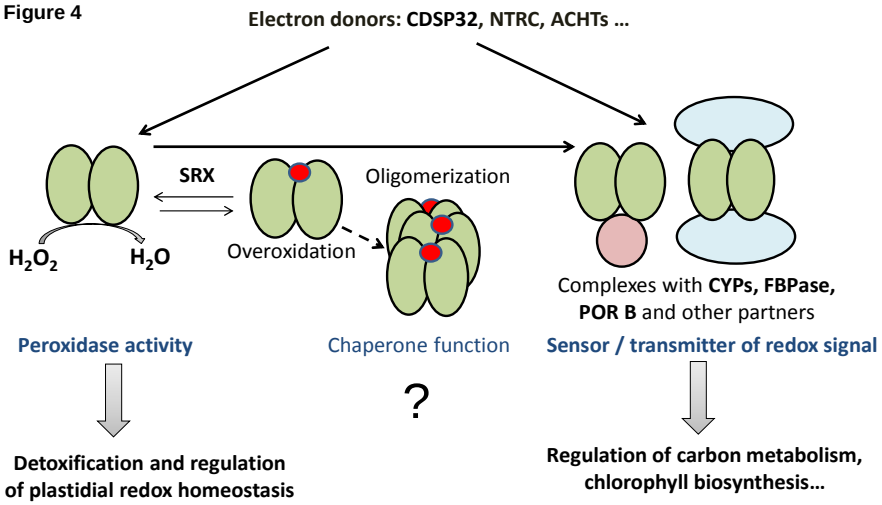

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Table 1: List of plastidial proteins (67) identified by co-immunoprecipitation of leaf extracts of *A. thaliana* plants using 2-Cys PRX antibodies and mass spectrometry. The table shows proteins, with the number of peptides, found only in elution fractions from Wt plants and a specific spectral count (SSC) higher than 3 (Specificity column) or with a SSC Wt/SSC 2cys*prx* ratio higher than 5 (indicated in the last column). Proteins in bold are known 2-Cys PRXs partners. * and †, indicate TRX partners in higher plants and unicellular photosynthetic organisms, respectively.

Entry UniProtKB	Gene	Protein name	Number of peptides	Specific spectral counting (SSC)	Specificity	Ratio SSC Wt / SSC 2cys*prx*
Antioxidant and redox homeostasis						
Q96291	At3g11630	**2-Cys peroxiredoxin A ***	14	86	Wt	
Q9C5R8	At5g06290	**2-Cys peroxiredoxin B ***	13	17	Wt	
O78310	At4g25100	Superoxide dismutase [Cu-Zn] 2 (SOD Cu-Zn) *	4	6	Wt	
P21276	At2g28190	Superoxide dismutase [Fe] 1 (SOD Fe)	4	5	Wt	
P42770	At3g54660	Glutathione reductase, chloroplastic (GR)	3	4	Wt	
Q8LE52	At5g16710	Glutathione S-transferase (DHAR3)	3	3	Wt	
Q96266	At2g47730	Glutathione S-transferase F8 (GST F8) *	3	3	Wt	
Q9C5U8	At5g63890	Histidinol dehydrogenase (HDH)	3	3	Wt	
Q9SGS4	At1g76080	**Thioredoxin-like protein CDSP32**	9	12		6
Q9LU86	At3g26060	Peroxiredoxin Q *	7	11		5.5
Q8LCT3	At4g29670	**Thioredoxin-like 2-2 (ACHT2)**	4	5		5
Amino acid metabolism						
Q93ZN9	At4g33680	LL-diaminopimelate aminotransferase (DAP-AT)	10	11	Wt	
Q9FZ47	At1g16880	ACT domain-containing protein ACR11	7	8	Wt	
P92981	At1g62180	5'-adenylylsulfate reductase 2 (APR 2) †	5	8	Wt	
Q94JQ4	At3g20390	Reactive Intermediate Deaminase A	6	7	Wt	
Q9SZX3	At4g24830	Argininosuccinate synthase (Citrulline--aspartate ligase) †	5	6	Wt	
P92980	At4g21990	5'-adenylylsulfate reductase 3 (APR 3) †	3	4	Wt	
Q9ZNZ7	At5g04140	Ferredoxin-dependent glutamate synthase 1 (Fd-GOGAT 1) * †	35	40		5.7
Q00218	At4g33510	Phospho-2-dehydro-3-deoxyheptonate aldolase 2	5	5		5
Protein folding						
Q93WL3	At4g25670	ATP-dependent Clp protease ATP-binding subunit CLPT1 *	9	12	Wt	
Q9SSA5	At3g01480	Peptidyl-prolyl cis-trans isomerase CYP38	5	6	Wt	
Q9LF37	At5g15450	Chaperone protein ClpB3	4	4	Wt	
Q945Q5	At2g30695	T11J7.9	3	3	Wt	
P82869	At3g15520	Peptidyl-prolyl cis-trans isomerase CYP37	2	3	Wt	
Q8S9L5	At5g55220	Trigger factor-like protein TIG	6	6		6
Chlorophyll synthesis						
Q42522	At3g48730	Glutamate-1-semialdehyde 2,1-aminomutase 2 (GSA 2) *	10	7	Wt	
P42799	At5g63570	Glutamate-1-semialdehyde 2,1-aminomutase 1 (GSA 1) *	8	4	Wt	
Q9LR75	At1g03475	Coproporphyrinogen-III oxidase 1 (CPO-I)	4	4	Wt	
P21218	At4g27440	**Protochlorophyllide reductase B (POR B)**	7	10		5
Photosynthetic metabolism						
Q9S726	At3g04790	Probable ribose-5-phosphate isomerase 3 * †	5	8	Wt	
P82538	At3g55330	PsbP-like protein 1	3	3	Wt	
O03042	Atcg00490	Ribulose bisphosphate carboxylase large subunit * †	36	7		7

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Q9FKW6	At5g66190	Ferredoxin--NADP reductase, leaf isozyme 1	14	15		5
Carbon metabolism						
P25851	At3g54050	**Fructose-1,6-bisphosphatase (FBPase)** *	11	14	Wt	
Q9SCY0	At5g51820	Phosphoglucomutase (PGM) †	9	10	Wt	
Q9C8P0	At4g16155	Dihydrolipoyllysine-residue acetyltransferase component 5 of pyruvate dehydrogenase complex	6	4	Wt	
Q9C6Z3	At1g30120	Pyruvate dehydrogenase E1 component subunit beta-2 *	3	4	Wt	
O24457	At1g01090	Pyruvate dehydrogenase E1 component subunit alpha-3 (PDH E1) *	12	12		12
Uncharacterized proteins						
Q8VY70	At1g62780	Putative uncharacterized protein	3	4	Wt	
P82658	At3g63540	Thylakoid lumenal 19 kDa protein (P19)	4	4	Wt	
Q9SR19	At3g04550	Rubisco accumulation factor 2 (RAF2)	4	4	Wt	
Q9S9M7	At1g16080	Putative uncharacterized protein	4	4	Wt	
Q9C685	At1g51100	Putative uncharacterized protein	3	3	Wt	
Q9M3C6	At3g55250	Putative calcium homeostasis regulator	3	3	Wt	
Q9ZUC1	At1g23740	Quinone oxidoreductase-like protein	7	9		9
Q9LXX5	At3g56650	PsbP domain-containing protein 6 (PPD6)	6	8		8
O80934	At2g37660	Uncharacterized protein	6	6		6
Q9LKR8	At5g28500	Rubisco accumulation factor 1 (RAF1)	3	5		5
Ribosomal proteins						
Q9LY66	At3g63490	50S ribosomal protein L1 (CL1)	6	7	Wt	
P51412	At1g35680	50S ribosomal protein L21 (CL21) *	3	4	Wt	
P42732	At5g14320	30S ribosomal protein S13 (CS13)	3	3	Wt	
Biotic and abiotic stress responses						
Q9LXC9	At5g09650	Soluble inorganic pyrophosphatase 6	3	3	Wt	
Q9M8M7	At1g80600	Acetylornithine aminotranferase *	3	3	Wt	
Q9SIF2	At2g04030	Heat shock protein 90-5	5	5		5
Assembly and repair of PSII						
O23403	At4g15510	PsbP domain-containing protein 1 (PPD1)	3	3	Wt	
Q9LR64	At1g03600	Photosystem II repair protein PSB27-H1	2	3	Wt	
Sulfur metabolism						
Q9LZ66	At5g04590	Sulfite reductase [ferredoxin] (AtSiR) * †	3	3	Wt	
P46309	At4g23100	Glutamate--cysteine ligase (GCS)	5	5		5
Lipid metabolism						
P52410	At5g46290	3-oxoacyl-[acyl-carrier-protein] synthase I (KAS1)	7	7	Wt	
Q9M1X2	At3g63170	Fatty-acid-binding protein 1 (FAP1)	4	4	Wt	
Nitrogen metabolism						
Q9ZST4	At4g01900	Nitrogen regulatory protein P-II homolog	3	4	Wt	
Q39161	At2g15620	Ferredoxin--nitrite reductase *	4	4	Wt	
Proteolysis						
Q9LJL3	At3g19170	Presequence protease 1 (PreP 1)	9	10	Wt	
Nucleotide metabolism						
P31166	At1g27450	Adenine phosphoribosyltranferase 1	3	4	Wt	
Secondary metabolism						
F4K0E8	At5g60600	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (ferredoxin) (ISPG)	4	4	Wt	
Development and cellular metabolism regulation						
Q9SZD6	At4g29060	Elongation factor Ts	9	9		9
Photorespiration						
P0DKC3	At5g36700	Phosphoglycolate phosphatase 1A	7	8		8