Genome-wide analysis and transcriptional regulation of the typical and atypical thioredoxins *in Arabidopsis thaliana*: Update

Kamel Chibani[1, 3‡], Boas Pucker[2], Karl-Josef Dietz[3], Amanda Cavanagh[1]

[1]School of Life Sciences, University of Essex, Colchester, UK.
[2]Department of Sciences, University of Cambridge, Cambridge, UK.
[3]Department of Biochemistry and Physiology of Plants, Faculty of Biology, University of Bielefeld, Germany.

Running title: Molecular evolution and transcriptional regulation of the Arabidopsis Trx subfamily.

Author ORCIDs
Kamel Chibani: 0000-0003-3229-400X.
Boas Pucker: 0000-0002-3321-7471.
Karl-Josef Dietz: 0000-0003-0311-2182.
Amanda Cavanagh: 0000-0001-5918-8093.

Author contributions
Kamel Chibani performed and designed the experiments and wrote the paper. Kamel Chibani, Boas Pucker, Karl Josef Dietz, Amanda Cavanagh analysed and interpreted the data and drafted the paper. Kamel Chibani, designed and directed the project. The final version was written through contribution of all authors.

Corresponding Author
Dr. Kamel Chibani
University of Essex
School of Biological Sciences
Wivenhoe Park (Colchester)
CO4 3SQ, UK
Email: kc20641@essex.ac.uk
Phone: 00/44/01206873726

Abstract

Thioredoxins (TRXs), a large subclass of ubiquitous oxidoreductases, are involved in thiol redox regulation. In the *Arabidopsis thaliana* genome 41 genes code for 18 typical, 23 atypical TRXs and, 6 thioredoxin reductases (TRs). The high number of atypical TRXs indicates special functions in plants that mostly await elucidation. Some new functions have recently emerged, illustrating the flexibility of TRX-dependent regulation, e.g. in the oxidative inactivation of the Calvin-Benson cycle, impacting photosynthetic carbon assimilation in fluctuating or changing light environment. An atypical class of thioredoxin called TRX-c was identified in the genomes of photosynthetic eukaryotes. Localized to the chloroplast, TRX-c displays peculiar atypical CPLC, CHLC and CNLC active sites. *In silico* analysis of the transcriptional regulations of *TRXs* revealed high expression of *TRX-c* in leaves and strong regulation under cold, osmotic, salinity and metal ion stresses.

Keywords: oxidoreductase, Arabidopsis, molecular evolution, regulation, thioredoxins.

Abbreviations: GRX: glutaredoxins, PDI: protein disulfide isomerases, TRX: thioredoxin, NTR: NADPH-thioredoxin reductase; FTR: ferredoxin-thioredoxin reductase.

1. Introduction

The thioredoxin superfamily consists of three main subclasses of oxidoreductases, the thioredoxins (TRX), glutaredoxins (GRX) and protein disulfide isomerases (PDI) (Holmgren et al., 1975; Meyer et al., 2005). The TRX subclass includes ubiquitous proteins of 12 to 14 kDa, essentially present in all prokaryotes and eukaryotes. TRXs serve as electron donors in a variety of cellular redox regulatory or metabolic dithiol-disulfide interchange reactions (Collet and Messens 2010). TRXs reductively dissolve disulfide bonds in target proteins with remarkable efficiency through their conserved redox-active canonical active site with a dithiol signature (W**C**GP**C**) (E°′~-270 mV) (Chibani et al., 2009). Their three-dimensional structure, the so-called TRX fold, consists of 4 β-sheets surrounded by 4 α-helices with a canonical WCGPC or non-canonical XCXXC catalytic motif located on a highly conserved fold at the periphery of the protein (Koh et al., 2008; Chibani et al., 2018). The catalytic cysteinyl residues of the active site form an internal disulfide bond. The N-terminal side position (C_N) (catalytic) is located in an exposed loop between strand β1 and helix α2 while the C-terminal (C_C) (resolving) is in helix α2 (Chibani et al., 2018). In the reduced dithiol state, the surface-exposed catalytic cysteinyl thiol of the active site attacks the disulfide bond in protein substrates with formation of an intermolecular disulfide intermediate. This mixed disulfide is subsequently attacked by the buried resolving cysteine (C_C), resulting in release of the reduced target protein and the oxidized Trx (Collet and Messens, 2010; Poole, 2015).

The XX residues between the cysteines of the active site are important factor for providing redox properties and substrate specificities toward the reducing systems and the target proteins (Chibani et al., 2012). Residues adjacent to the CXXC motif, such as the conserved tryptophan (W) in canonical Trxs can have a large effect on the protein stability, pKa of the nucleophilic cysteine and the redox potential of the protein (LeMaster et al., 1997; Roos et al., 2010). In human Trx, an aspartate (D) is responsible for the pH dependence for dimer formation (Andersen et al., 1997).

According to their active site, TRXs divide into two main clades, the typical (WCGPC active site) and the atypical (XCXXC active site) TRXs, which in turn separate into several classes according to their primary structure and cellular compartmentation. The **typical TRX**-f, -m, -x, -y, -z and a group of **atypical TRXs** (TRX-like, Lilium CDSP32, NTRC and FTR) localize in plastids (Meyer et al., 2005; Chibani et al., 2009; Chibani et al., 2012). The typical TRX-o type is located in the mitochondria (Laloi et al., 2001), while the h-types are mainly

cytosolic or mitochondrial (Gelhaye, 2004; Renard et al., 2011; Hägglund et al., 2016). TRXs regulate a wide range of enzymes involved in photosynthesis, carbohydrate metabolism and photorespiration (Chibani et al., 2010). They have the ability to reduce disulfide bonds on target enzymes/proteins that perform reduction reactions. In the chloroplast, mitochondria and cytosol, TRXs provide electrons to peroxiredoxins (PRXs), methionine sulfoxide reductases (MSRs) and glutathione peroxidases (GPXs) (Gelhaye, 2004; Chibani et al., 2012; Vaseghi et al., 2018).

TRXs also catalyze post-translational modifications (PTMs), including transnitrosylation (Wu et al., 2010), denitrosylation (Benhar et al., 2008; Wu et al., 2010), and deglutathionylation of specific proteins (Greetham et al., 2010; Kehr et al., 2011). In plants, typical and atypical TRXs are involved in regulating biological processes such as lipid, carbohydrate and nitrogen metabolism. The classical processes under control of TRXs are photosynthesis, seed germination and acclimation to environmental constraints (Chibani et al., 2010; Nikkanen and Rintamäki, 2019). Even here, new players such as thiol-dependent activity regulation of β-carbonic anhydrase are presently described (Dreyer et al., 2020).

Despite the intensive investigation, a clear and precise classification distinguishing the typical TRXs with WCGPC motif and atypical TRXs with XCXXC, including their transcriptional regulation, is not complete. Based on sequence analysis, we present here a precise overview of the Arabidopsis TRXs and identify an atypical TRX-c class containing a characterised TRX-DCC1 (Ginalski et al., 2004; Zhang et al., 2018). We discuss the distribution of the TRX-c with CPLC, CHLC and CNLC active sites in vascular plants, unicellular algae and mosses. This *in silico* analysis highlights the putative subcellular localization and summarizes the relative transcript abundance in different Arabidopsis tissues in dependence on abiotic stresses conditions.

2. Materials and Methods

2.1 Bioinformatic protein analysis

The Arabidopsis typical and atypical TRX protein sequences were retrieved from the *A. thalina* whole genome database (version 1.1) at the U.S. Department of Energy Joint Genome Institute (JGI) (www.arabidopsis.org/). All accession numbers of the protein sequences used in this article are also found in the UniProt database (www.uniprot.org/). The curated amino acid sequences were used to query other genome sequence annotations using BLASTP program using Phytozome database (phytozome.jgi.doe.gov/pz/portal.html). The genome sequences of

the other eukaryotes are available at the Phytozome database and prokaryotes at the bacteria kazusa database (bacteria.kazusa.or.jp/cyanobase/).

2.2 Subcellular localization

The subcellular localization of the proteins was predicted using Predotar (urgi.versailles.inra.fr/predotar/french.html) (Small et al., 2004), TargetP (www.cbs.dtu.dk/services/TargetP/) (Emanuelsson et al., 2007) and WoLF PSORT (wolfpsort.seq.cbrc.jp/) (Horton et al., 2007). Bouchnak et al. (2019), confirmed the plastidial subcellular localization of the TRX proteins (Envelope of the chloroplast) after a quantitative proteome (Mass spectrometry) study.

2.3 Phylogenetic analysis

The amino acid sequence alignments were done using CLUSTALW and imported into the Molecular Evolutionary Genetics Analysis (MEGA) software version 5 (Tamura et al., 2011). Phylogenetic analyses were conducted using the neighbor-joining (NJ) method implemented in MEGA version 5, with the pairwise deletion option for handling alignment gaps, and with the Poisson correction model for distance computation. Bootstrap tests comprised 1,000 replicates and are depicted with branch lengths proportional to the calculated phylogenetic distances. The phylogenetic trees are shared on the iTOL server (Letunic and Bork, 2016), and are available at itol.embl.de/tree/. All the sequences are available in Supplemental Material 1.

2.4 *In silico* analysis of gene expression data

To assess the abundance of *A. thaliana TRX* transcripts in plant organs under control and stress conditions, we used the Affymetrix ATH1 GeneChip data set available via Gene Expression Omnibus (GEO) (www.ncbi.nlm.nih.gov/geo). The GSE5630, GSE5631, GSE5633, and GSE5634 datasets contains the microarray data of *A. thaliana* plant organs (leaf, root, shoots/stem and seeds/siliques) described by Schmid et al. (2005). The GSE5621, GSE5622, and GSE5624 datasets contains the abiotic stress (cold, osmotic, and drought) responses as described by Kilian et al. (2007). The GSE108751 dataset depicts the transcript data of metal ion (aluminium, copper, zinc, cadmium) and salinity stress (NaCl), described by Zhao et al. (2007). The heatmap was generated using the Python library matplotlib (Hunter, 2007) and seaborn (Waskom et al., 2020). The transcript abundance of each gene was

standardized using log and z-score transformation. A form of normalization that is particularly useful when comparing samples from diverse treatments/tissue backgrounds.

3. Results and Discussion

3.1 The classification and subcellular localization of TRXs and TRs

The *A. thaliana* genome encodes 41 TRXs (18 typical, and 23 atypical TRXs) and, 6 TRs (Table 1). A phylogenetic tree was constructed representing the typical TRXs with canonical WCGPC active site and the atypical TRXs with non-canonical WCXXC active site (Fig. 1).

The typical TRXs comprises seven classes (Fig. 1) (Table 1). These includes the class of the mitochondrial TRX-o class, the cytosolic TRX-h and TDX classes and the chloroplastic TRX-z; -x, -y and -m classes (Fig. 1). These classes have been extensively studied in photosynthetic organisms (Chibani et al., 2009; Belin et al., 2015). TRX-f, -h, and -o are evolutionarily close to eukaryotic sequences, whereas TRX-m, -x, -y and -z closely relate to prokaryotic sequences (Meyer et al., 2005; Belin et al., 2015). The number of isoforms and the active sites of the typical TRXs are represented in Table 1.

The atypical TRXs comprises nine different classes: The cytosolic TRX-h-like proteins class, the CDSP32 (Chloroplastic drought-induced stress protein of 32 KDa (Broin and Rey, 2003) class, and the plastidic ACHT proteins class (Atypical Cys His-rich Trx) formerly named Lilium TRXs. TRX-Lilium family was first identified in *Lilium longiflorum* based on homology with a thioredoxin proteins (Chibani et al., 2009). The chloroplastic TRX-like3 and the chloroplastic HCF164 (High Chlorophyll Fluorescence 164) (Chibani et al., 2009) forms two independent classes. Moreover, the cytosolic Clot and the nucleoredoxin (NRX) classes cluster separately. The *A. thaliana* genome exploration and the phylogenetic analysis identified a class of TRX that we called TRX-c (Table 1) represented by three isoforms, TRX-c1, -c2 and -c3 with 16 to 24% of similarity. These isoforms form the independent cluster (Fig. 1B) and localize in the envelope (Bouchnak et al., 2019). However, TRX-c1 (TRX-DCC1) has also been shown to interact with carbonic anhydrase (CA2) in the mitochondria (Zhang et al., 2018) suggesting that this protein may be dual-targeted as it was also found to be well represented in the chloroplast envelope (Bouchnak et al., 2019), and comparison of the subcellular functions remain an important avenue for future research. The TRX-c active sites contain the motifs CPLC, CHLC, CNLC, respectively (Table 1; Suppl. Fig. 1). TRX-c3 displays shorter N- and C-terminal sequences (Supplemental Fig. 1). The TRX-c class is present in eukaryotes only (Table 2). All three isoforms are found among cormophytes, though their distribution varies, e.g., *Glycine max* lacks TRX-c2, however TRX-c1 is present in duplicate form, while *Nicotiana tabacum* possesses two copies of each TRX-c isoforms. The fern *Selaginella moellendorffii*

only possesses the isoform TRX-c3, while TRX-c2 is absent in mosses and algae (Table 2). This distribution pattern may indicate the ability for mutual complementation. This hypothesis should be explored. The number of isoforms and the active sites of the atypical TRXs are represented in Table 1.

Table 1: Major features of *A. thaliana* TRXs and TRs.

	Class	Gene name[a]	Gene models[b]	Protein ID[c]	Length[d] (aa)	Putative localization[e]	Redox centers[f]
Typical TRX	**TRX-f**	f1	At3g02730	Q9XFH8	178	P	WCGPC
		f2	At5g16400	Q9XFH9	185	P	WCGPC
	TRX-h	h1	At3g51030	P29448	114	C	WCGPC
		h2	At5g39950	Q38879	133	M/MR	WCGPC
		h7	At1g59730	Q9XIF4	129	C	WCGPC
		h8	At1g69880	Q9CAS1	148	C	WCGPC
		h9	At3G08710	Q9C9Y6	140	P/M	WCGPC
	TRX-m	m1	At1g03680	O48737	179	P	WCGPC
		m2	At4g03520	Q9SEU8	186	P	WCGPC
		m3	At2g15570	Q9SEU7	173	P	WCGPC
		m4	At3g15360	Q9SEU6	193	P	WCGPC
	TRX-o	o1	At2g35010	O64764	194	M/MR	WCGPC
		o2	At1g31020	Q93VQ9	159	M	WCGPC
	TDX	TDX	At3g17880	Q8VWG7	380	P	WCGPC
	TRX-x	x	At1g50320	Q8LD49	182	P	WCGPC
	TRX-y	y1	At1g76760	Q6NPF9	172	P	WCGPC
		y2	At1g43560	Q8L7S9	167	P	WCGPC
	TRX-z	z	At3g06730	Q9M7X9	183	P	WCGPC
Atypical TRX	**ACHT (Lilium)**	ACHT1	At4g26160	Q8LEK4	221	P	WCGSC
		ACHT2	At4g29670	Q8LCT3	236	P	WCASC
		ACHT3	At2g33270	O22779	273	P	GCGGC
		ACHT4	At1g08570	A8MQQ7	244	P	GCGGC
		ACHT5	At5g61440	Q9XFI1	186	P	GCGGC
		ACHT6	At1g07700	Q9C5C5	204	P	ACGSC
	CDSP32	CDSP32	At1g76080	Q9SGS4	302	P	HCGPC
	Clot	Clot	At5g42850	Q9FMN4	134	C	WCPDC
	CXXS	CXXS1	At1g11530	Q8LDI5	118	C	WCIPS
		CXXS2	At2g40790	Q8GXV2	154	C	WCLPS
	TRX-c	c1	At5g50100	Q8W485	214	P/M	DCPLC
		c2	At1g24095	Q9LR85	213	P	VCHLC
		c3	At1g52590	Q9SSR1	172	P	VCNLC
	HCF	HCF164	At4g37200	O23166	261	P	WCEVC
	TRX-h like	h3	At5g42980	Q42403	118	C	WCPPC
		h4	At1g19730	Q39239	119	C	WCPPC
		h5	At1g45145	Q39241	118	C/S	WCPPC
		h10	At3g56420	Q9LXZ8	154	C	WCVPC
	TRX-L3 (Like 3)	L3.1	At5g06690	Q9FG36	214	P	WCRKC
		L3.2	At5g04260	Q8VZT6	192	P	WCRKC
		L3.3	At3g53220	Q8LCH9	126	P	WCGVC
	NRX	NRX1	At1g60420	O80763	578	N/C	WCGPC/WCPPC
		NRX2	At4g31240	Q8VZQ0	392	N/C	WCPPC/WCPPF
Thioredoxin reducatse (TR)	Flavo-thioredoxin						
	NTR	NTRA	At2g17420	Q39242	383	C	CAVC
		NTRB	At4g35460	Q39243	375	M/C	CAVC
		NTRC	At2g41680	O22229	529	P	ACAIC/TCGPC
	FTR	FTRA.1	At5g23440	Q9FHL4	182	P	RCDVAIK
		FTRA.2	At5g08410	Q8LBP6	184	P	RCDIAVK
		FTRB	At2g04700	Q9SJ89	164	P	WNCPC

[a,b] The gene name and model come from the Tair website (https://www.arabidopsis.org/).
[c]The protein name come from Tair website and verified in UniProt website (https://www.uniprot.org/).
[d]Length of the proteins in amino acids (aa).
[e]Localizations are based on three prediction programs (Predotar (http://urgi.versailles.inra.fr/predotar/frenP.html), TargetP (http://www.cbs.dtu.dk/services/TargetP/)), Wolfpsort (http://wolfpsort.seq.cbrc.jp/). C: cytosol; M: mitochondria; MR: microsome; S: secreted; P: plastid. The subcellular and subplastidial localizations were confirmed from the quantitative proteome (Mass spectrometry) study of Bouchnak et al., (2019) Mol Cell Proteomics 18; 1285–1306.
[f]The protein ID is available at the UniProt website.
[g]The redox center was verified in UniProt website.
All sequences are available in supplemental material 1.

Table 2: Presence of TRX-c isoforms in photosynthetic organisms

	TRX-c1	TRX-c2	TRX-c3
Cormophyte			
Monocot			
Oryza sativa	1	1	1
Zea mays	2	1	1
Brachypodium distachyon	1	1	1
Dicot			
Arabidopsis thaliana	1	1	1
Beta vulgaris	1	1	1
Glycine max	2	0	1
Nicotiana tabacum	2	2	2
Populus trichocarpa	1	1	1
Fern			
Selaginella moellendorffii	0	0	1
Moss			
Marchantia polymorpha	1	0	1
Algae			
Chlamydomonas reinhardtii	1	0	1
Cyanobacteria			
Synechocystis sp PCC 6803	0	0	0
Nostoc punctiforme ATCC 29133	0	0	0

Sequences have been retrieved from Phytozome v12.1, a Joint Genome Institute database.
All sequences are available in supplemental material 2.

The thioredoxin reductases (TRs) group consists of three NTRs (NTR: NADPH-TRX reductase A, B and C) and three FTRs (Ferredoxin (FDX)-dependent-TRX reductase) (Table 1). NTR distribute among subcellular compartments: cytosolic NTRA, cytosolic and mitochondrial NTRB, and the plastidic NTRC (Chibani et al., 2009; Kang et al., 2019; Bouchnak et al., 2019). FTRs are present in plastids of photosynthetic organism and are clearly distinguished from NTRs by their dependence on reduced FDX as electron donor (Keryer et al., 2004). The FTR genes encode variable α-chains (FTRA.1 and FTRA.2, also called FTRV1 and FTRV2) and catalytic ß-chain subunits (FTRB, also called FTRC) (Nikkanen and Rintamaki, 2014; Buchanan et al., 2016). NTRC and FDX/FTR/TRX are separate systems, although NTRC interacts with both TRXs and FTRs *in vivo* (Jacquot et al., 2009). Both systems are vital to chloroplast, and more general to plastid function and plant growth (Schurmann and Buchanan, 2008). The 3D structure of the FTR differs from the three isoforms of NTR, but they all reduce the typical TRXs (-f, -m, -x, -y, and -z) (Yoshida and Hisabori, 2017). This is a clear case of convergent functional evolution (Jacquot et al., 2009).

3.2 Expression of *TRXs and TRs* in Arabidopsis organs

The expression of the Arabidopsis *TRX* and *TR* genes was studied at the transcript level using Arabidopsis transcriptome data available in GEO (www.ncbi.nlm.nih.gov/geo/) (Schmid et al., 2005). Measured amounts of 41 *TRX* and 6 *TR* transcripts identified in the genome were compiled from roots, stems, seeds/siliques and mature leaves. While the typical *TRX-f1*, *-f2*, *-m2*, *-m4*, *-x*, *-y2* and, *–z* are highly expressed in leaves, *TRX -m3*, *-h2*, and *-h7* are highly expressed in roots (Fig. 2). *TRX-m*, *-f*, *-x* and *-y* play a role in the redox regulation of photosynthetic metabolism. Light-dependent reduction occurs via the FDX/FTR system (Bohrer et al., 2012; Yoshida and Hisabori, 2017). TRX-f, and -m take part in activation and deactivation of the Calvin–Benson cycle in dependence of the reductive pressure built up in the photosynthetic electron transport, whereas TRX-x and -y were proposed to mainly participate in the control of enzymes involved in the antioxidant response (Michelet et al., 2013; Geigenberger et al., 2017; Vaseghi et al., 2018; Telman et al., 2020). TRX-f1 deficiency impairs light-dependent reductive activation of ADP-glucose pyrophosphorylase (AGPase) and starch turnover (Thormählen et al., 2013). Lack of TRX-z induces a severe albino phenotype indicating that the function of this TRX in chloroplast transcription is not redundant with other plastidial TRXs (Arsova et al., 2010; Meng et al., 2010). *TRX-m4* is involved in alternative

photosynthetic electron transport (Courteille et al., 2013), and the simultaneous deficiency of *TRX-m1*, *-m2*, and *-m4* resulted in impaired photosystem II biogenesis (Wang et al., 2013).

TRX-h2, *-h7* and *-h9* are higher in roots than in stem, leaf and seeds (Fig. 2). The distinct isoforms of TRX-h that differ in spatial distribution and kinetic properties suggest that they play different roles in plant development (Renard et al., 2011). TRX-h2 is targeted to the mitochondria which plays an important role in the activation of the alternative oxidase (AOX) and the deactivation of the photorespiratory metabolism enzyme such as the glycine decarboxylase L subunit (GDC-L) (Gelhaye et al., 2004; da Fonseca-Pereira, 2019; Hou et al., 2021). A T-DNA insertion mutation revealed that TRX-h9 was required for growth and development (Meng et al., 2010).

Mitochondrial *TRX-o1* and *-o2* accumulate more in roots than in leaves and seeds/siliques. Further, *At-trx-o1* T-DNA mutants have been used to unravel the regulatory mechanisms by which the mitochondrial TRX system regulates TCA cycle enzymes, photorespiration, and mitochondrial electron transport pathways *in vivo* (Daloso et al., 2015; Florez-Sarasa et al., 2019). The lack of TRX-o1 impacts stomata development and aperture but not photosynthesis (Sánchez-Guerrero et al., 2021).

Figure 2 show that the atypical *TRXs* have a diverse expression pattern in different plant organs. *ACHT1, ACHT3* and *ACHT4* transcripts are abundant in seeds/siliques, whereas *ACHT2* is higher in leaves. This result is in accordance with the semiquantitative RT-PCR (sqRT-PCR) analysis of the *AtACHT* family genes (Dangoor et al., 2009). *CDSP32* transcript is 2 to 4 fold higher in leaves than roots and siliques. The *Solanum tuberosum CDSP32*, participates in plastid defence against oxidative damage and its expression is related to leaf age and stress conditions (Broin and Rey, 2003).

The *TRX-h-like* class exhibits a very diverse pattern of gene expression. *TRXs- h3* and *-h5* are highly expressed in roots whereas the *TRX-h4* and *CXXS2* are high in seeds/siliques. Montrichard et al., (2003) reported a differential pattern of expression of pea cytosolic *TRXs-h3* and *-h4* in early seedlings and seeds and suggested that the proteins encoded were closely linked to germination. Members of the atypical *TRX-L3* class are highly expressed in leaves and less expressed in roots, stems and seeds. On the other hand, some transcript variations were observed for *Clot* and *HCF164* classes. They exhibit their highest expression in roots and leaves, respectively.

The *HCF164* gene encodes a protein which is located inside the chloroplast and is anchored to the thylakoid membrane at its lumenal side. The *hcf164* T-DNA mutant of *A. thaliana* is deficient specifically in the biogenesis of the cytochrome b_6f complex (Bechtold et

al., 1993; Lennartz et al., 2001). Interestingly, within the *TRX-c* class, *TRX-c1* is highly expressed in seeds/siliques whereas, *TRX-c3* exhibits a higher expression in leaves and is weakly expressed in roots and stems. Concerning the *NRX* class, *NRX1 has* a relatively high expression in roots and stems, *NRX2* was found highly expressed in roots and seeds (Fig. 2), whereas both display low accumulation in leaves. The *Atnrx1* mutant plant shows a reduced pollen fertility phenotype (Marchal et al., 2014), whereas the *Atnrx2* knockout plant develops less trichomes compared with the wild type and the *Atnrx1* mutant (Lee et al., 2020; Kang et al., 2020).

For the *TR* group (*NTRCs* and *FTRs*), the chloroplastic *NTRC, FTRA.1, FTRA.2 and FTRB* preferentially expressed in leaf mesophyll whereas the cytosolic *NTRA* has a preferred expressional activity in roots. The RT-PCR expression analysis of the Arabidopsis *NTRA* showed a ubiquitous regulation in all the organs including roots, rosette leaves, stems, flower buds and flowers (Reichheld et al., 2007). *NTRC* and *FTRAs* are light-dependently and preferentially expressed in green tissues, but they are also detected in non-photosynthetic tissues of *A. thaliana*, where they localize to other forms of plastids (Ferrández et al., 2012; Kirchsteiger et al., 2012; Balsera et al., 2013). They are involved in maintaining the redox homeostasis of plastids in non-photosynthetic organs (Kirchsteiger et al., 2012; Balsera et al., 2013). The *ftra* T-DNA mutants of *A. thaliana* exhibit a delay in emergence of the floral stem and plant growth (Keryer et al., 2004).

In immature tomato fruits, NTRC downregulation decreased transient starch accumulation. This effect led to a subsequent decrease in soluble sugars in ripe fruits. This metabolic change could be linked to a decrease in the redox-activation state of ADP-Glc pyrophosphorylase and soluble starch synthase. In addition to the capacity to regenerate the 2-CysPRX, NTRC acts as a central hub regulating carbon metabolism and redox balance in heterotrophic fruits which affect tomato fruit size and quality (Hou et al., 2019). NTRC overexpression induced enhanced starch accumulation in tobacco and Arabidopsis leaves (Toivola et al., 2013; Ancín et al., 2019; Guinea Diaz et al., 2020). This part of the redox regulatory network of the chloroplast was recently modelled in mathematical simulations and proved the predominance of the NTRC-branch over the TRX-branch in regeneration of 2-CysPRX (Gerken et al. 2020). This mathematical model also quantitatively assessed the capacity of 2-CysPRX to oxidize the chloroplast fructose-1,6-bisphosphatase via TRX-f upon switching off the photosynthetic electron transport chain by, e.g., darkening (Gerken et al., 2020).

3.2 *TRXs and TRs* expression under abiotic stresses

3.2.1 Gene expression under osmotic, cold, and drought stresses

The TRX superfamily is involved in several biological processes, including biogenesis of plastids, translation and photosynthesis, but also plays a profound antioxidant function with significant implications for acclimation to stressful conditions (Chibani et al., 2010). To tentatively assess this antioxidant role, *TRXs and TRs* expression were examined under osmotic, cold, and drought stresses using the AtGenExpress dataset available in GEO (Kilian et al., 2007) (Fig. 3). While the typical *TRX-h7* is repressed in root under osmotic stress *TRX-y2* and *-z* types are repressed in shoots. As well as *TRX-h8* is overexpressed under drought conditions only in roots, *TRX-h9* is overexpressed in shoots and roots under cold stress condition. It is well documented in several species, that *TRXs-h* is often induced in response to drought and osmotic stresses (Watkinson et al., 2008; Schürmann and Jacquot, 2000; Sun et al., 2010). In maize, the *TRXs-h* are upregulated under osmotic stresses such as treatment with polyethlene glycol (PEG) and abscisic acid (ABA) (Koscy et al., 2004). *TRXs-h* are up and downregulated in adapted and acclimated drought-stressed *Solanum tuberosum* (Watkinson et al., 2008). These seemingly erratic responses are far from being understood. They may be connected to synergistic and antagonistic regulation not only of antioxidant activity by electron donation to glutathione peroxidase-like proteins and PRXs (Dietz 2016, Meyer et al. 2021), but also reflect linkages to yet unknown signalling pathways, e.g., by controlling redox state of transcription factors and their subcellular partitioning (Dietz, 2014).

From Figure 3, the atypical *TRX* group seems to be slightly more responsive than the typical *TRXs* under abiotic stress. *ACHT4* is overexpressed upon osmotic stress, whereas *ACHT5* is repressed in response to cold and osmotic stresses in the same time. In the TRX-c class, the abundance of *TRX-c1* increases in shoots under cold and osmotic stresses, whereas that of the *CXXS1* decreases only in roots under cold stress. Interestingly *CDSP32* expression is high in osmotic stress and repressed upon cold stress. Its mRNA and protein accumulate in large quantities upon induction by oxidative and drought stresses, showing an increase in hydrogen peroxide and hyperoxidation 2-CysPRX (Broin and Rey 2003). Overexpressing *CDSP32* enhances drought, salt, and oxidative stress tolerance in *A. thaliana* (Eleasad et al., 2020). *TRX-h5* is overexpressed in response to osmotic and drought stresses in shoots. *TRX-h5* displays a strong increase in response to various abiotic (osmotic, salt, drought, UV-B and wounding) and biotic stresses in shoots (Laloi et al., 2004). In the tolerant *Triticum aestivum* cultivar, *TRX-h5* abundance increased under drought conditions, while it decreased in the sensitive cultivar (Hajheidari et al., 2007). *NRX1* and *NRX2* are overexpressed under osmotic

stress in shoots and roots, respectively. Mutant *NRX1* plants displayed reduced catalase activity and were hypersensitive to oxidative stress (Kneeshaw et al., 2007). NRXs have been shown to play an interesting role as a protective mechanism of antioxidant systems controlling the status of ROS-scavenging enzymes such as catalase, and probably that of APX, MDHAR and DHAR (Calderón et al., 2018).

3.2.2 Salinity and metal stress

Ion metals are divided into two groups: the redox active [Iron (Fe), Cu (copper) and Zn (Zinc)] and the non-redox active [Al (Aluminium), Arsenic (As), Cd (Cadmium), and Hg (Mercury)] (Valko et al., 2015). Ion metals at excess concentrations are toxic compounds for cells. Cd, Hg and Cu are examples of highly toxic heavy metal ions. As and Al are less toxic but often more bioavailable than the mentioned heavy metals and NaCl exhibits inhibitory effect at high millimolar concentrations (>50 mM for pea, depending on species) (Arif et al., 2016). Al^{3+} ions cause severe damage to the roots of plants growing in acid soil, accentuating nutrient deficiency, and increasing their sensitivity to drought stress (Kochian et al., 2004). Growth inhibition concerns roots and shoots, due to inhibition of meristematic activities and because ions metals have negative impacts on the shoot yield of crop plants (Gupta et al., 2013; Sharma et al. 2021).

In *Chlamydomonas reinhardtii***,** typical *TRX-h* and *-m* types are highly regulated under heavy metals (Cd and Hg) (Lemaire et al., 1999). However, few information is available on the reactivity of the other members of the typical and atypical Trxs of plants upon exposure to metal ions. To elucidate *TRX* reactivities under rhizotoxic stresses (Al, Cd, Cu and NaCl), *TRXs* expression was examined using the AtGenExpress dataset available in GEO (Zhao et al., 2007) (Fig. 4). In photosynthetic organisms, salinity and metal stresses are poorly investigated. Typical and atypical *TRXs* seem to be more reactive to rhizotoxic stressor than other abiotic stresses. Toxic Al exposure overexpress distinct and specific sets of transcripts such as *TRX-f1, -h7, -h8, -m1, -m3, -z* and *TDX*. Li et al. (2010) reported that overexpression of a *TRX-h* confers increased tolerance to Al in barley roots. Cd treatment stimulates *TRX-o1*, *-o2* and *-h9* accumulation and represses *TRX-f1* and *-h8*. In *Chlamydomonas reinhardtii*, upon Cd treatment *TRX-m* and *-h* is induced and the TRX-h protein was inhibited, while the TRX-m activity remained unchange (Lemaire et al., 1999). Cu stimulates the level of *TRXs -f2*, *-h2*, *-h7*, *-m2 -m4*, and *-y2*, while it did not induce any decrease in gene expression. TRX proteins are suggested to act by reducing free Cu ions through regulating the binding capacity of the reduced form of TRX Cu ions (Hellinga et al., 1991). In plants, Cu plays a crucial role in photosynthesis,

respiration, oxidative stress responses, cell wall metabolism, and hormone perception. However, Cu excess is toxic and induces inhibition of photosynthesis, pigment synthesis and oxidative stress (Burkhead et al. 2009) by mediating the Fenton reaction producing hydroxyl radicals (Sharma and Dietz 2009). In mammals, metal ions such as auranofin and metal complexes (zinc and cadmium acetate, cisplatin, tributyltin) are TRX and TR inhibitors (Ouyang, et al., 2018). Metal ions exhibit a high affinity to TRX and TR, which inhibit their activities. The soft base properties of the TRX cysteine and the selenocysteine (Sec) of the TR confer confer high reactivity with metal ions. The deprotonated forms of the selenol of Sec in TR and cysteine of the TRX can serve as soft bases to react with soft acids. In mitochondria, auranofin, chloro(triethylphosphine) gold, and aurothiomalate act as inducers of permeability transition, a membrane potential decrease and provoke the formation of hydrogen peroxide (H_2O_2) (Bragadin et al., 2004).

Unlike Cu, NaCl induces only the repression of a set of genes such as *TRXs -f2*, *-h2*, *-h7*, *-m3*, *-o1*, *-o2, -y2*, *-z* and *TDX* in roots (Fig. 4). The gene expression of typical *TRXs* under salinity conditions depended on species, NaCl concentration and test system. It is documented that *PsTRX-m1*, *-m2*, *-m4* and *–f* of salt-sensitive *Pisum sativum* and *ZmTRX-m1* mRNAs from maize increase during germination under salinity condition. Moreover, overexpression of *PsTRXs-m1*, *-m2* and *-f* conferred resistance to salinity (Fernández-Trijueque et al., 2012). Salt stress provoked an accumulation of *PsTRXs -f*, *-m1* and *-o1* transcripts (Martí et al., 2011; Fernández-Trijueque et al., 2012). Decreased amounts of *AtTRX-o1* influences stomatal development and aperture under saline growth conditions (Sánchez-Guerrero et al., 2021). Salinity induces an increase of *TRX-h* at transcriptional and post-transcriptional levels during germination and early seedling growth of wheat (Cazalis et al. 2006).

Atypical *TRXs* plays a profound role in response to ionic stresses. Aluminium toxicity limits crop quality and yield by inducing an oxidative stress response (Matiello et al., 2014). *ACHT2*, *ACHT4*, *h5*, *h10*, *NRX1* and *NRX2* are highly expressed upon Al treatment whereas, *CXXS1*, *NTRA* and *NTRC* are strongly repressed (Figure 4). The response of plants upon cadmium treatment is still poorly studied, however our *in silico* analysis showed that the level of *ACHT3*, *CXXS1*, *CXXS2*, *HCF164*, *h10*, *L3.1*, *NRX2* transcripts was significantly decreased. Cu treatment significantly stimulated the level of *Clot*, *CXXS2*, *TRX-c3*, *TRX-h3*, *TRX-h10*, and *L3.1* transcripts. Song et al. (2013) reported that excess Cu activates the accumulation of L3.3 protein, suggesting an important role of the atypical Trx in the detoxification of Cu and maintaining cellular redox homeostasis in *Oryza sativa* roots. Under NaCl treatment, *ACHT4*, *TRX-c3*, *TRX-h5*, and *NRX1* are highly repressed, whereas *NTRA* and *NTRC* are slightly

repressed. This pattern of gene expression suggests that the cytosolic, secreted, nuclear and plastidial atypical Trxs likely are involved in tuning directly or indirectly the molecular response to NaCl stress response. Overexpressing *Tamarix hispida* ThTrx5 confers salt tolerance to *A. thaliana* by activating stress response signals (Luan et al., 2020). NTRA overexpression confers oxidative stress tolerance and the *AtNTRC* knockout mutants develop a hypersensibility to saline stress (Serrato et al., 2004; Cha et al., 2014).

These examples of transcriptional changes in response to abiotic stresses provide circumstantial evidence for a substantial role of TRX and TRX-like proteins in tuning the plant responses and acclimation, e.g. by controlling the redox state of transcription or translation factors (Dietz, 2014; Moore et al., 2016).

4. Conclusions

The present phylogenetic and transcript abundance analyses demonstrate that TRX constitutes a wide subfamily which is expressed under abiotic stresses. The atypical TRX-c class is conserved among cormophytes. Future wet lab (enzyme assays) and *in silico* work including mathematical modelling needs to establish the specificity of the atypical TRX class as a redox transmitter and regulator in the reductive or oxidative branch. Its physiological role *in planta* remains to be investigated. Additionally, the expression of the *TRXs* and *TRs* under ion stress suggest that the sulfhydryl group (thiol) of the active site may function as ligands for several metal ions, suggesting a possible role for TRXs in defense mechanisms against rhizotoxic stress in photosynthetic organisms.

5. Acknowledgments

This work was supported by funding from the DFG and Bielefeld University to K-J D funding (DI 346). We thank the Center for Biotechnology (CeBiTec) at Bielefeld University for providing an environment to perform the computational analyses.

6. Conflicts of Interest

The authors declare no conflict of interests.

7. Figure legends

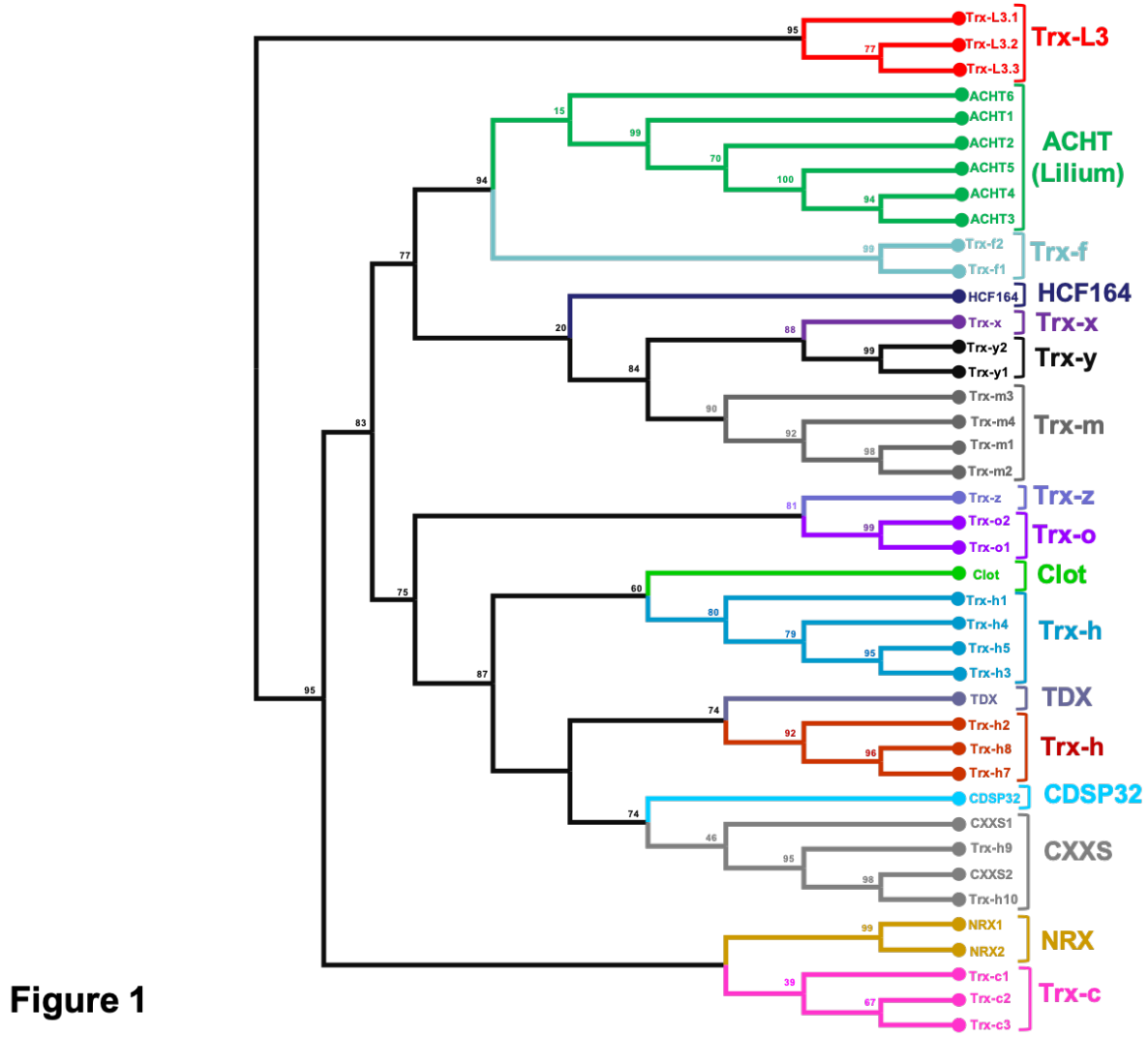

Figure 1

Figure 1. Maximum likelihood phylogenies from *A. thaliana* thioredoxins (TRX). (a) Representation of the typical TRX amino acid sequences with a WCGPC active site. (b) Represent the atypical TRX amino acid sequences with a XCXXC active site. The analysis was performed using MEGA 5 program with bootstrap test (1000 times) using the neighbour-joining method. Branch lengths are proportional to phylogenetic distances. For clarity, the protein names have been shortened. The coloured branch indicates the different class.

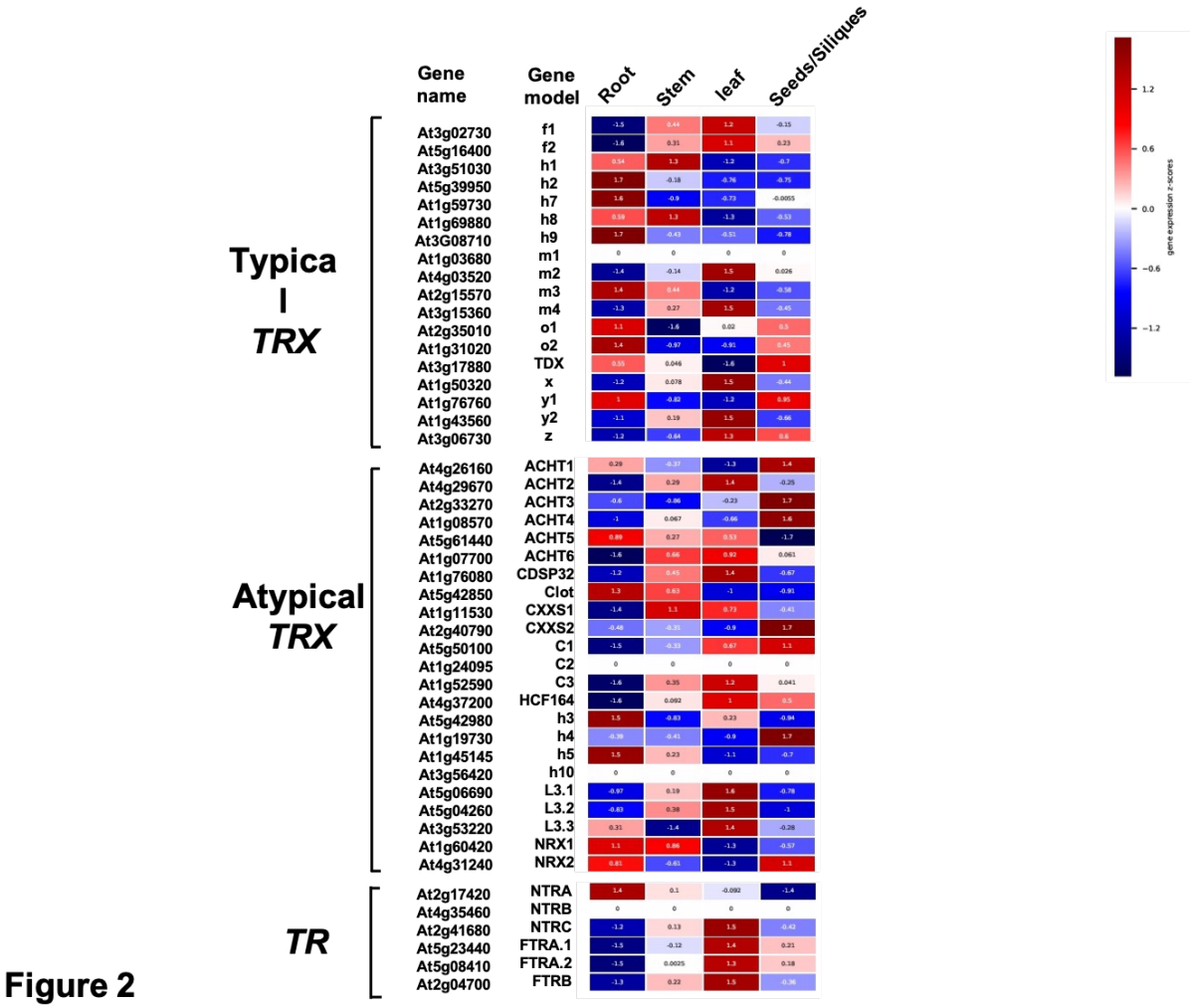

Figure 2

Figure 2. Expression profiles of typical and atypical TRX genes in different plant organs (leaves, roots, seeds/siliques and shoots). Heatmap was constructed from the microarray experimental data (generated using Affymetrix ATH1 GeneChip arrays) of Arabidopsis development (AtGenExpress Developmental Expression Atlas), described by Schmid et al. (2005). Intensity values of replicates in the heap-map were averaged and z-score transformed. Red and blue indicate higher and lower expression values, respectively.

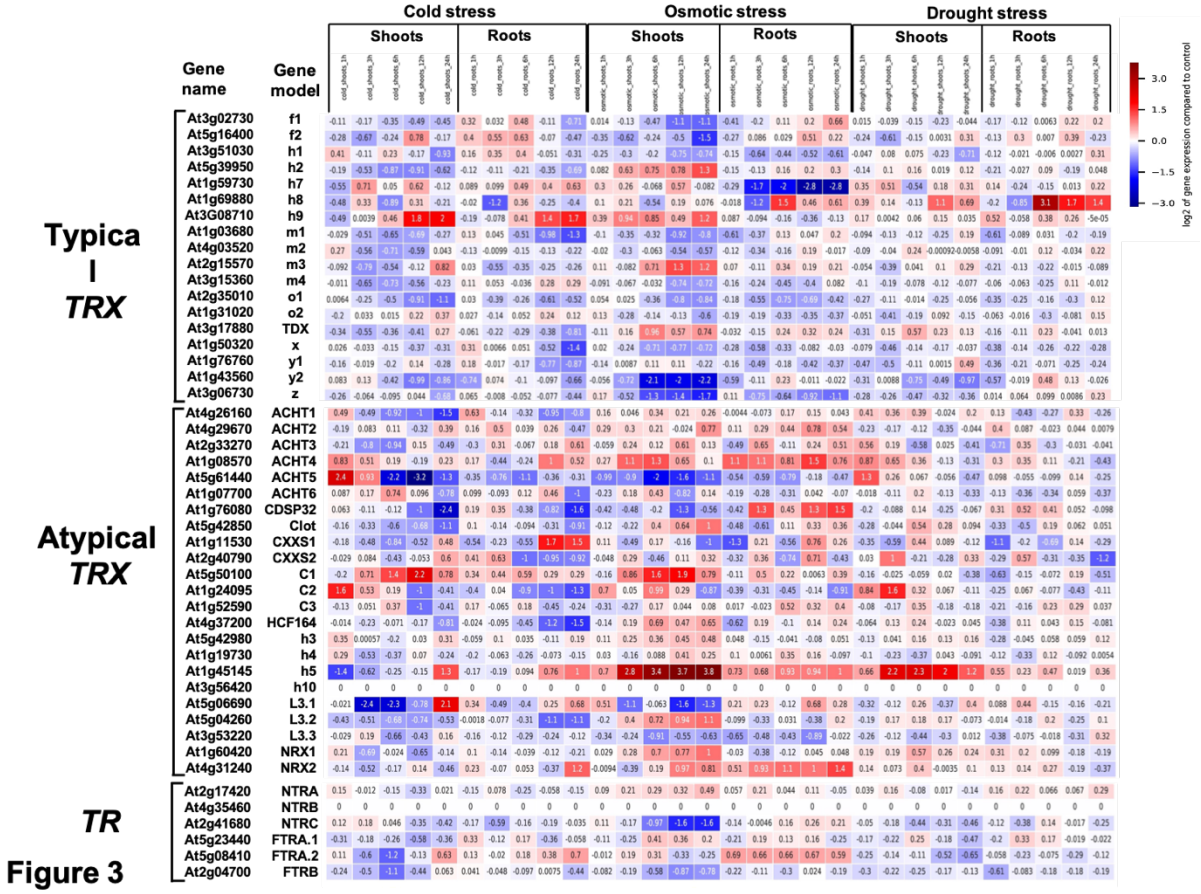

Figure 3. Expression profiles of typical and atypical TRXs under some abiotic stresses in shoots and roots. Heatmap was constructed from the microarray experimental data (generated using Affymetrix ATH1 GeneChip arrays). Treatment and number of replicates were described by Kilian et al. (2007). Intensity values of two biological replicates in the heatmap were averaged and normalized based on the control condition (treatment/control). Control represents non-stressed shoots and roots. Red and blue indicate higher and lower expression values, respectively.

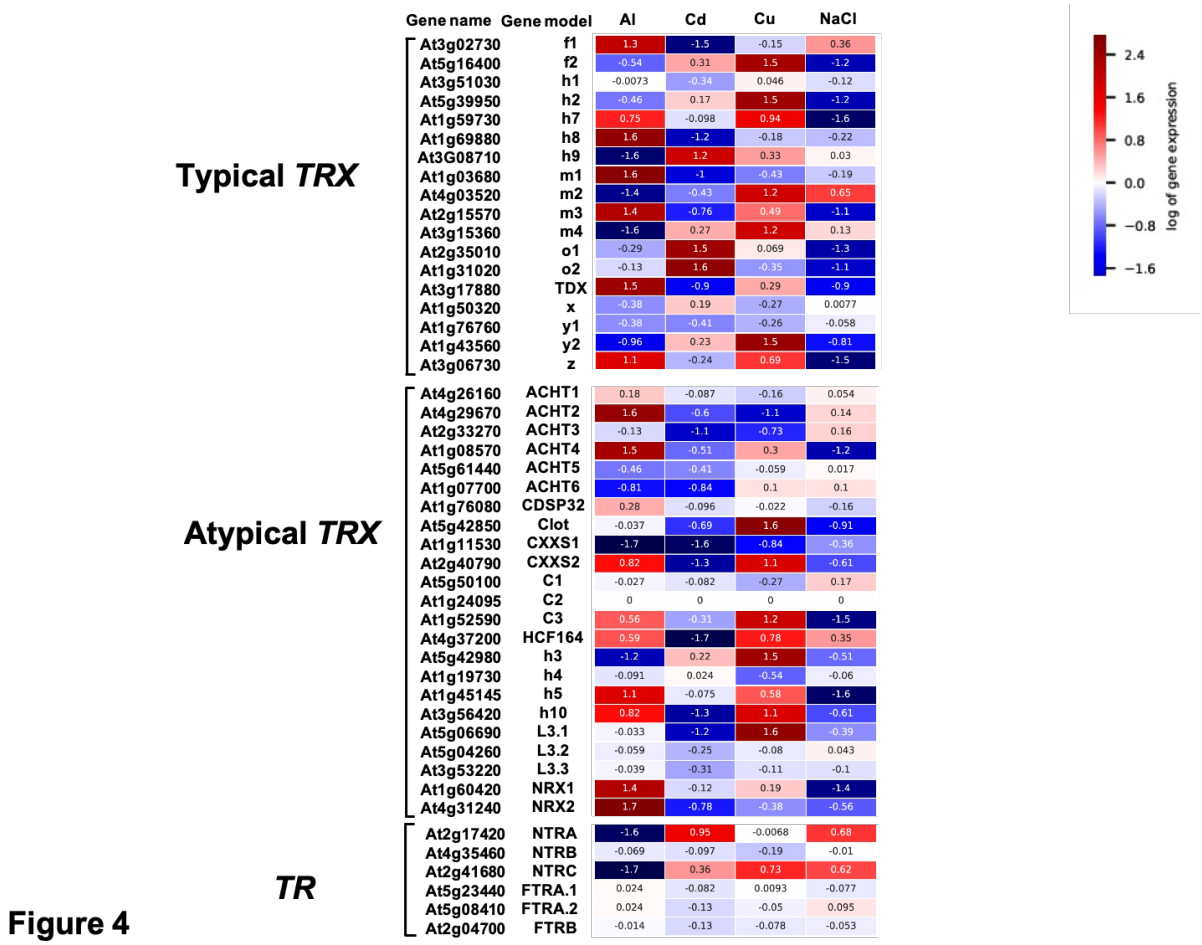

Figure 4

Figure 4. Expression profiles of typical and atypical genes under ionic stresses in Arabidopsis root. Heatmap was constructed from the microarray experimental data (generated using Affymetrix ATH1 GeneChip arrays). Treatment and number of replicates were described by Zhao et al. (2007). Intensity values of three biological replicates in the heatmap were averaged and z-score transformed based on the control condition. Control represents non-stressed roots. Red and blue indicate higher and lower expression values, respectively

8. Supplementary material

All the sequences used in this study have been added in the pdf file entitled supplemental material 1 and 2.

9. References

Ancín M, Larraya L, Fernández-San Millán A, Veramendi J, Burch-Smith T, Farran I (2019) NTRC and Thioredoxin f Overexpression Differentially Induces Starch Accumulation in Tobacco Leaves. *Plants* (Basel) **26**, 543.

Andersen JF, Sanders DA, Gasdaska JR, Weichsel A, Powis G, Montfort WR (1997) Human thioredoxin homodimers: regulation by pH, role of aspartate 60, and crystal structure of the aspartate 60 --> asparagine mutant. *Biochemistry* **18**, 13979-88.

Arif N, Yadav V, Singh S, Singh S, Ahmad P, Mishra R K., Sharma S, Tripathi DK, Dubey NK., Chauhan DK. (2016) Influence of High and Low Levels of Plant-Beneficial Heavy Metal Ions on Plant Growth and Development. *Frontiers in Environmental Science* **4**, 69.

Arsova B, Hoja U, Wimmelbacher M, Greiner E, Ustün S, Melzer M, Petersen K, Lein W, Börnke F (2010) Plastidial thioredoxin z interacts with two fructokinase-like proteins in a thiol-dependent manner: evidence for an essential role in chloroplast development in Arabidopsis and *Nicotiana benthamiana*. *The Plant Cell* **22**, 1498–1515.

Balsera M, Uberegui E, Susanti D, Schmitz RA, Mukhopadhyay B, Schürmann P, Buchanan BB (2013) Ferredoxin:thioredoxin reductase (FTR) links the regulation of oxygenic photosynthesis to deeply rooted bacteria. *Planta* **237**, 619-35.

Bechtold N, Ellis, J, and Pelletier, G (1993) In planta *Agrobacterium*–mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *C. R. Acad. Sci.* **316,** 1194–1199.

Belin C, Bashandy T, Cela J, Delorme-Hinoux V, Riondet C, Reichheld JP (2015) A comprehensive study of thiol reduction gene expression under stress conditions in Arabidopsis thaliana. *Plant Cell Environ* **38**, 299-314.

Benhar M, Forrester MT, Hess DT, Stamler JS (2008) Regulated protein denitrosylation by cytosolic and mitochondrial thioredoxins. *Science* **23**, 1050-4.

Bohrer AS, Massot V, Innocenti G, Reichheld JP, Issakidis-Bourguet E, and Vanacker H (2012) New insights into the reduction systems of plastidial thioredoxins point out the unique properties of thioredoxin z from Arabidopsis. *J Exp Bot* **63**, 6315–6323

Bouchnak I, Brugière S, Moyet L, Le Gall S, Salvi D, Kuntz M, Tardif M, Rolland N (2019) Unraveling Hidden Components of the Chloroplast Envelope Proteome: Opportunities and Limits of Better MS Sensitivity. *Mol Cell Proteomics* **18**, 1285-1306.

Bragadin M, Scutari G, Folda A, Bindoli A, Rigobello MP (2004) Effect of metal complexes on thioredoxin reductase and the regulation of mitochondrial permeability conditions. *Ann N Y Acad Sci* **1030**, 348-54.

Broin M, Rey P (2003) Potato plants lacking the CDSP32 plastidic thioredoxin exhibit overoxidation of the BAS1 2-cysteine peroxiredoxin and increased lipid Peroxidation in thylakoids under photooxidative stress. *Plant Physiol* **132**, 1335-43

Buchanan BB (2106) The Path to Thioredoxin and Redox Regulation in Chloroplasts. *Annu Rev Plant Biol* **29**, 1-24.

Burkhead JL, Reynolds KAG, Abdel-Ghany SE, Cohu CM, Pilon M (2009) Copper homeostasis. *New Phytol* **182**, 799–816.

Calderón A, Francisca Sevilla F, Jiménez A (2018) Redox Protein Thioredoxins: Function Under Salinity, Drought and Extreme Temperature Conditions. *Antioxidants and Antioxidant Enzymes in Higher Plants*, 123-162.

Cazalis R, Pulido P, Aussenac T, Pérez-Ruiz JM, Cejudo FJ (2006) Cloning and characterization of three thioredoxin h isoforms from wheat showing differential expression in seeds. *J Exp Bot* **57**, 2165–2172.

Cha JY, Kim JY, Jung IJ, Kim MR, Melencion A, Alam SS, Yun DJ, Lee SY, Kim MG, Kim WY (2014) NADPH-dependent thioredoxin reductase A (NTRA) confers elevated tolerance to oxidative stress and drought. *Plant Physiol Biochem* **80**,184-91.

Chibani K, Wingsle G, Jacquot JP, Gelhaye E, Rouhier N (2009) Comparative genomic study of the thioredoxin family in photosynthetic organisms with emphasis on *Populus trichocarpa*. *Mol Plant* **2**, 308-322.

Chibani K, Couturier J, Selles B, Jacquot JP, Rouhier N (2010) The chloroplastic thiol reducing systems: dual functions in the regulation of carbohydrate metabolism and regeneration of antioxidant enzymes, emphasis on the poplar redoxin equipment. *Photosynth Res* **104**, 75-99.

Chibani K, Tarrago L, Gualberto JM, Wingsle G, Rey P, Jacquot JP, Rouhier N (2012) Atypical thioredoxins in poplar: the glutathione-dependent thioredoxin-like 2.1 supports the activity of target enzymes possessing a single redox active cysteine. *Plant Physiol* **159**, 592-605.

Chibani K, Saul F, Didierjean C, Rouhier N, Haouz A (2018) Structural snapshots along the reaction mechanism of the atypical poplar thioredoxin-like2.1 *FEBS Lett* **592**, 1030-1041.

Collet JF, and Messens J (2010) Structure, Function, and Mechanism of Thioredoxin Proteins. *Antioxidants & Redox Signaling* **13**, 1205-1216.

Courteille A, Vesa S, Sanz-Barrio R, Cazalé AC, Becuwe-Linka N, Farran I, Havaux M, Rey P, Rumeau D (2013) Thioredoxin m4 controls photosynthetic alternative electron pathways in Arabidopsis. *Plant Physiology* **161**, 508–520.

da Fonseca-Pereira P, Daloso DM, Gago J, de Oliveira Silva FM, Condori-Apfata JA, Florez-Sarasa I, Tohge T, Reichheld JP, Nunes-Nesi A, Fernie AR, Araújo WL (2019) The Mitochondrial Thioredoxin System Contributes to the Metabolic Responses Under Drought Episodes in Arabidopsis. *Plant Cell Physiol* **60**, 213-229.

Daloso, DM, Müller K, Obata T, Florian A, Tohge T, Bottcher A, Riondet C, Bariat L, Carrari F, Nunes-Nesi A, et al (2015) Thioredoxin, a master regulator of the tricarboxylic acid cycle in plant mitochondria. *Proc. Natl. Acad. Sci. USA* **112**, 1392–1400.

Dangoor I, Peled-Zehavi H, Levitan A, Pasand O, Danon A (2009) A small family of chloroplast atypical thioredoxins. *Plant Physiol* **149**, 1240-1250.

Dietz K-J (2014) Redox regulation of transcription factors in plant stress acclimation and development. *Antioxidants & Redox Signaling* **21**, 1356-1372.

Dietz KJ (2016) Thiol-based peroxidases and ascorbate peroxidases: why plants rely on multiple peroxidase systems in the photosynthesizing chloroplast? *Molecules and Cells* **39**, 20-25.

Dreyer A, Schackmann A, Kriznik A, Chibani K, Wesemann C, Vogelsang L, Beyer A, Dietz KJ (2020) Thiol Redox Regulation of Plant β-Carbonic Anhydrase. *Biomolecules* **10**, 1125.

Elasad M, Ahmad A, Wang H, Ma L, Yu S, Wei H (2020) Overexpression of CDSP32 (GhTRX134) Cotton Gene Enhances Drought, Salt, and Oxidative Stress Tolerance in Arabidopsis. *Plants (Basel)* **19**, 1388.

Emanuelsson O, Brunak S, von Heijne G, Nielsen H (2007) Locating proteins in the cell using TargetP, SignalP and related tools. *Nat Protoc* **2**, 953-71.

Ferrández J, González M, Cejudo FJ (2012) Chloroplast redox homeostasis is essential for lateral root formation in Arabidopsis. *Plant Signal Behav* **7**, 1177-9.

Fernández-Trijueque J, Barajas-López JD, Chueca A, Cazalis R, Sahrawy M, Serrato AJ (2012) Plastid thioredoxins f and m are related to the developing and salinity response of post-germinating seeds of Pisum sativum. *Plant Sci* **188**, 82–88.

Florez-Sarasa I, Obata T, Del-Saz NF, Reichheld JP, Meyer EH, Rodríguez-Concepción M, Ribas-Carbó M, Fernie, AR (2019) The lack of mitochondrial thioredoxin TRXo1 affects in vivo alternative oxidase activity and carbon metabolism un der different light conditions. *Plant Cell Physiol* **60**, 1–13.

Geigenberger P, Thormählen I, Daloso DM, Fernie AR (2017)The Unprecedented Versatility of the Plant Thioredoxin System. *Trends Plant Sci* **22**, 249-262.

Gelhaye E, Rouhier N, Gerard J, Jolivet Y, Gualberto J, Navrot N, Ohlsson PI, Wingsle G, Hirasawa M, Knaff DB, Wang H, Dizengremel P, Meyer Y, Jacquot JP (2004) A specific form of thioredoxin h occurs in plant mitochondria and regulates the alternative oxidase. *Proc Natl Acad Sci USA* **101**, 14545-14550.

Gerken M, Kakorin S, Chibani K, Dietz KJ (2020) Computational simulation of the reactive oxygen species and redox network in the regulation of chloroplast metabolism. PLoS Computational Biology 16 (1), e1007102.

Ginalski K, Kinch L, Rychlewski L, Grishin NV (2004) DCC proteins: a novel family of thiol-disulfide oxidoreductases. *Trends Biochem Sci* **7**, 339-42.

Greetham D, Vickerstaff J, Shenton D, Perrone GG, Dawes IW, Grant CM (2010) Thioredoxins function as deglutathionylase enzymes in the yeast *Saccharomyces cerevisiae*. *BMC Biochem* **14**, 11-13.

Guinea Diaz M, Nikkanen L, Himanen K, Toivola J, Rintamäki E (2020) Two chloroplast thioredoxin systems differentially modulate photosynthesis in Arabidopsis depending on light intensity and leaf age. *Plant J* **104**, 718-734.

Gupta DK, Corpas FJ, Palma JM (2013) Heavy metal stress in plants. *Springer, Berlin, Heidelberg* **12**, 242.

Hägglund P, Finnie C, Yano H, Shahpiri A, Buchanan BB, Henriksen A, Svensson B (2016) Seed thioredoxin h. *Biochim Biophys Acta* **1864**, 974-82.

Hajheidari M, Eivazi A, Buchanan BB, Wong JH, Majidi I, Salekdeh GH (2007) Proteomics uncovers a role for redox in drought tolerance in wheat. *J Proteom Res* **6**, 1451–1460.

Hellinga HW, Caradonna JP, Richards FM (1991) Construction of new ligand binding sites in proteins of known structure: II. Grafting of a buried transition metal binding site into Escherichia coli thioredoxin. *J Mol Biol* **222**, 787-803.

Holmgren A, Söderberg BO, Eklund H and Brändén CI (1975) Three-dimensional structure of *Escherichia coli* thioredoxin-S2 to 2.8 A resolution. *Proc Natl Acad Sci USA* **72**, 2305-9.

Horton P, Park KJ, Obayashi T, Fujita N, Harada H, Adams-Collier CJ, Nakai K (2007) WoLF PSORT: protein localization predictor. *Nucleic Acids Res* **35**, 585-7.

Hou LY, Ehrlich M, Thormählen I, Lehmann M, Krahnert I, Obata T, Cejudo FJ, Fernie AR, Geigenberger P (2019) NTRC Plays a Crucial Role in Starch Metabolism, Redox Balance, and Tomato Fruit Growth. *Plant Physiol* **181**, 976-992.

Hou LY, Lehmann M, Geigenberger P (2021) Thioredoxin *h*2 and *o*1 Show Different Subcellular Localizations and Redox-Active Functions, and Are Extrachloroplastic Factors Influencing Photosynthetic Performance in Fluctuating Light. *Antioxidants (Basel)* **10**, 705.

Hunter JD (2007) Matplotlib: A 2D Graphics Environment. *Computing in Science & Engineering* **9**, 90 – 95.

Jacquot JP, Eklund H, Rouhier N, Schürmann P (2009) Structural and evolutionary aspects of thioredoxin reductases in photosynthetic organisms. *Trends Plant Sci* **14**, 336-43.

Kang Z, Qin T, Zhao Z (2019) Thioredoxins and thioredoxin reductase in chloroplasts: A review. *Gene* **706**, 32-42.

Kang CH, Park JH, Lee ES, Paeng SK, Chae HB, Hong JC, Lee SY (2020) Redox-Dependent Structural Modification of Nucleoredoxin Triggers Defense Responses against. *Int J Mol Sci* **21**, 9196.

Kehr S, Jortzik E, Delahunty C, Yates JR 3rd, Rahlfs S, Becker K (2011) Protein S-glutathionylation in malaria parasites. *Antioxid Redox Signal* **15**, 2855-65.

Keryer E, Collin V, Lavergne D, Lemaire S, Issakidis-Bourguet E (2004) Characterization of Arabidopsis Mutants for the Variable Subunit of Ferredoxin:thioredoxin Reductase. *Photosynth Res* **79**, 265-74.

Kilian, J, Whitehead D, Horak J, Wanke D, Weinl S, Batistic O, et al. (2007). The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses: AtGenExpress global abiotic stress data set. *Plant J* **50**, 347–363.

Kirchsteiger K, Ferrández J, Pascual MB, González M, Cejudo FJ (2012) NADPH thioredoxin reductase C is localized in plastids of photosynthetic and nonphotosynthetic tissues and is involved in lateral root formation in Arabidopsis. *Plant Cell* **24**, 1534-48.

Kneeshaw S, Keyani R, Delorme-Hinoux V, Imrie L, Loake GJ, Le Bihan T, Reichheld JP, Spoel SH (2017) Nucleoredoxin guards against oxidative stress by protecting antioxidant enzymes. *Proc Natl Acad Sci U S A* **114**, 8414-8419.

Kochian LV, Hoekenga OA, Pineros MA (2004): How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorous efficiency. *Annu Rev Plant Biol* **55**, 459-493.

Kocsy G, Kobrehel K, Szalaia G, Duviau MP, Buzás Z Galiba G (2004) Abiotic stress-induced changes in glutathione and thioredoxin h levels in maize. *Env Exp Bot* **52**, 101-112

Koh CS, Navrot N, Didierjean C, Rouhier N, Hirasawa M, Knaff DB, Wingsle G, Samian R, Jacquot JP, Corbier C, Gelhaye E (2008) An atypical catalytic mechanism involving three cysteines of thioredoxin. *J Biol Chem* **283**, 23062-23072.

Laloi C, Rayapuram N, Chartier Y, Grienenberger JM, Bonnard G, Meyer Y (2001) Identification and characterization of a mitochondrial thioredoxin system in plants. *Proc Natl Acad Sci USA* **98**, 14144-14149.

Laloi C., Mestres-ortega D., Marco Y., Meyer Y. & Reichheld J (2004) The Arabidopsis cytosolic thioredoxin h5 gene induction by oxidative stress and its W-box-mediated response to pathogen elicitor 1. *Plant Physiology* **134**, 1006–1016.

Lee EU, Park JH, Hwang GY, Chi YH, Kang CH, Chae HB, Paeng SK, Wi SD, Bae SB Phan KAT, Lee SY (2020) Nucleoredoxin2 (NRX2) Promotes Jasmonate-Mediated Trichome Formation in Arabidopsis. *Journal of Plant Biology* **63**, 95–50.

LeMaster DM, Springer PA, Unkefer CJ (1997) The role of the buried aspartate of *Escherichia coli* thioredoxin in the activation of the mixed disulfide intermediate. *J Biol Chem* **28**, 29998-30001.

Lemaire S, Keryer E, Stein M, Schepens I I, Issakidis-Bourguet E, G rard-Hirne C, Miginiac-Maslow M, Jacquot JP (1999) Heavy-metal regulation of thioredoxin gene expression in *chlamydomonas reinhardtii*. *Plant Physiol* **120**, 773-8.

Lennartz, K, Plucken, H, Seidler A, Westhoff P, Bechtold N, & Meierhoff K (2001) HCF164 encodes a thioredoxin-like protein involved in the biogenesis of the cytochrome b(6)f complex in Arabidopsis. *The Plant Cell* **13**, 2539–2551.

Letunic I, Bork P (2016) Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Research* **44**, 242–245.

Li Q, Niu H, Yin J, Shao H, Niu J, Ren J, Li Y, Wang X (2010) Transgenic barley with overexpressed PTrx increases aluminum resistance in roots during germination. *J Zhejiang Univ Sci B* **11**, 862-870.

Luan J, Dong J, Song X, Jiang J, Li H (2020) Overexpression of *Tamarix hispida* ThTrx5 Confers Salt Tolerance to Arabidopsis by Activating StressResponse Signals. *Int J Mol Sci* **21**,1165.

Marchal C, Delorme-Hinoux V, Bariat L, Siala W, Belin C, Saez-Vasquez J, Riondet C, Reichheld JP (2014) NTR/NRX define a new thioredoxin system in the nucleus of Arabidopsis thaliana cells. *Mol Plant* **7**, 30-44.

Martí MC, Florez-Sarasa I, Camejo D, Ribas-Carbó M, Lázaro JJ, Sevilla F, Jiménez A (2011) Response of mitochondrial thioredoxin PsTrxo1, antioxidant enzymes, and respiration to salinity in pea (Pisum sativum L.) leaves. *J Exp Bot* **62**, 3863–3874.

Mattiello L, Begcy K, da Silva FR, Jorge RA, Menossi M (2014) Transcriptome analysis highlights changes in the leaves of maize plants cultivated in acidic soil containing toxic levels of Al(3+). *Mol Biol Rep* **41**, 8107-8116.

Meng L, Wong JH, Feldman LJ, Lemaux PG, Buchanan BB (2010) A membrane-associated thioredoxin required for plant growth moves from cell to cell, suggestive of a role in intercellular communication. *Proc Natl Acad Sci U S A* **23**, 3900-5.

Meyer AJ, Dreyer A, Ugalde JM, Feitosa-Araujo E, Dietz KJ, Schwarzländer M (2021) Shifting paradigms and novel players in Cys-based redox regulation and ROS signaling in plants-and where to go next. *Biological Chemistry* **402**, 399-423.

Meyer Y, Reichheld JP, Vignols F (2005) Thioredoxins in Arabidopsis and other plants. *Photosynth Res* **3**, 419-33.

Michelet L, Zaffagnini M, Morisse S, et al. (2013). Redox regulation of the Calvin–Benson cycle: something old, something new. *Frontiers in Plant Science* **4**, 470.

Montrichard F, Renard M, Alkhalfioui F, Duval FD, Macherel D (2003) Identification and differential expression of two thioredoxin h isoforms in germinating seeds from pea. *Plant Physiol* **132**, 1707-1715.

Moore M, Gossmann N, Dietz KJ (2016) Redox regulation of cytosolic translation in plants. *Trends Plant Sci.* **21**, 388-397.

Nikkanen L, Rintamäki E (2014) Thioredoxin-dependent regulatory networks in chloroplasts under fluctuating light conditions. *Philos Trans R Soc Lond B Biol Sci* **3**, 369.

Nikkanen L, Rintamäki E (2019) Chloroplast thioredoxin systems dynamically regulate photosynthesis in plants. *Biochem J* **15**, 1159-1172.

Ouyang Y, Peng Y, Li J, Holmgren A, Lu J (2018) Modulation of thiol-dependent redox system by metal ions via thioredoxin and glutaredoxin systems. *Metallomics* **10**, :218-228.

Poole LB (2015) The basics of thiols and cysteines in redox biology and chemistry. *Free Radic Biol Med* **80**, 148-157.

Reichheld JP, Khafif M, Riondet C, Droux M, Bonnard G, Meyer Y (2007) Inactivation of thioredoxin reductases reveals a complex interplay between thioredoxin and glutathione pathways in Arabidopsis development. *Plant Cell* **19**, 1851-65.

Renard M, Alkhalfioui F, Schmitt-Keichinger C, Ritzenthaler C, Montrichard F (2011) Identification and characterization of thioredoxin h isoforms differentially expressed in germinating seeds of the model legume Medicago truncatula. *Plant Physiol* **155**, 1113-26.

Roos G, Geerlings P, Messens J (2010) The conserved active site tryptophan of thioredoxin has no effect on its redox properties. *Protein Sci* **19**, 190-194.

Sánchez-Guerrero A, Nadal M, Florez-Sarasa I, Ribas-Carbó M, Vallarino JG, De Brasi-Velasco S, Fernie AR, Flexas J, Jiménez A, Sevilla F (2021) Decreased Levels of Thioredoxin *o1* Influences Stomatal Development and Aperture but Not Photosynthesis under Non-Stress and Saline Conditions. *Int J Mol Sci* **21**, 1063.

Schmid M, Davison TS, Henz SR, Pape UJ, Demar M, Vingron M, Schölkopf B, Weigel D, Lohmann JU (2005) A gene expression map of Arabidopsis thaliana development *Nat Genet* **37**, 501-6.

Schürmann P, Buchanan BB (2008) The ferredoxin/thioredoxin system of oxygenic photosynthesis. *Antioxid Redox Signal* **10**, 1235-74.

Schürmann, P, Jacquot, JP (2000) Plant thioredoxin systems revisited. Annu Rev Plant Physiol. *Plant Mol Biol* **51**, 371-400.

Serrato AJ, Pérez-Ruiz JM, Spínola MC, Cejudo FJ (2004) A novel NADPH thioredoxin reductase, localised in the chloroplast, which deficiency causes hypersensitivity to abiotic stress in Arabidopsis thaliana. *J Biol Chem* **279**, 43821–43827.

Sharma SS, Dietz KJ (2009) The relationship between metal toxicity and cellular redox imbalance. *Trends Plant Sci.* **14**, 43-50.

Sharma SS, Kumar V, Dietz KJ (2021) Emerging Trends in Metalloid-Dependent Signaling in Plants. *Trends Plant Sci.* **26**, 452-471.

Small I, Peeters N, Legeai F, Lurin C (2004) Predotar: A tool for rapidly screening proteomes for N-terminal targeting sequences. *Proteomics* **4**, 1581-90.

Song YF, Cui J, Zhang HX, Wang GP, Zhao FJ, Shen ZG (2013) Proteomic analysis of copper stress responses in the roots of two rice (*Oryza sativa* L.) varieties differing in Cu tolerance. *Plant Soil* **366**, 647–658.

Sun L, Ren H, Liu R, Li B, Wu T, Sun F, Liu H, Wang X, Dong H (2010) An h-type thioredoxin functions in tobacco defense responses to two species of viruses and an abiotic oxidative stress. *Mol Plant Microbe Interact* **23**, 1470-85.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731-2739.

Telman W, Liebthal M, Dietz KJ (2020) Redox regulation by peroxiredoxins is linked to their thioredoxin-dependent oxidase function. *Photosynthesis Research* **145**, 31-41.

Thormählen I, Ruber J, von Roepenack-Lahaye E, Ehrlich SM, Massot V, Hümmer C, Tezycka J, Issakidis-Bourguet E, Geigenberger P (2013) Inactivation of thioredoxin f1 leads to decreased light activation of ADP-glucose pyrophosphorylase and altered diurnal starch turnover in leaves of Arabidopsis plants. *Plant, Cell and Environment* **36**, 16–29.

Toivola J, Nikkanen L, Dahlström KM, Salminen TA, Lepistö A, Vignols HF, Rintamäki E (2013) Overexpression of chloroplast NADPH-dependent thioredoxin reductase in Arabidopsis enhances leaf growth and elucidates in vivo function of reductase and thioredoxin domains. *Front Plant Sci* **8**, 4:389.

Valko M, Jomova K, Rhodes CJ, Kuča K, Musílek K (2016) Redox- and non-redox-metal-induced formation of free radicals and their role in human disease. *Arch Toxicol* **90**, 1-37.

Vaseghi MJ, Chibani K, Telman W, Liebthal MF, Gerken M, Schnitzer H, Mueller SM, Dietz KJ (2018). The chloroplast 2-cysteine peroxiredoxin functions as thioredoxin oxidase in redox regulation of chloroplast metabolism. *Elife* **12**, 7.

Wang P, Liu J, Liu B, et al. (2013) Evidence for a role of chloroplastic m-type thioredoxins in the biogenesis of photosystem II in Arabidopsis. *Plant Physiology* **163**, 1710–172.

Waskom M, Botvinnik O, Ostblom J, Lukauskas S, Hobson P, Gelbart M, Gemperline DC, Augspurger T, Halchenko Y, Cole JB et al., (2020) mwaskom/seaborn: v0. 10.0. Zenodo. http://doi.org/10.5281/zenodo.3629446.

Watkinson JI, Hendricks L, Sioson AA, Heath LS, Bohnert HJ, Grene R (2008) Tuber development phenotypes in adapted and acclimated, drought-stressed *Solanum tuberosum* ssp. andigena have distinct expression profiles of genes associated with carbon metabolism. *Plant Physiol Biochem* **46**, 34-45.

Wu C, Liu T, Chen W, Oka S, Fu C, Jain MR, Parrott AM, Baykal AT, Sadoshima J, Li H (2010) Redox regulatory mechanism of transnitrosylation by thioredoxin. *Mol Cell Proteomics* **9**, 2262-75.

Yoshida K, Hisabori T (2017) Distinct electron transfer from ferredoxin-thioredoxin reductase to multiple thioredoxin isoforms in chloroplasts. *Biochem J* **4**, 1347-1360.

Zhang, H., Zhang, T. T., Liu, H., Shi, Y., Wang, M., Bie, X. M., Li, X. G., & Zhang, X. S. (2018) Thioredoxin-Mediated ROS Homeostasis Explains Natural Variation in Plant Regeneration. *Plant physiology* **176**, 2231–2250.

Zhao CR, Ikka T, Sawaki Y, Kobayashi Y, Suzuki Y, Hibino T, Sato S, Sakurai N, Shibata D, Koyama H (2009) Comparative transcriptomic characterization of aluminum, sodium chloride, cadmium and copper rhizotoxicities in *Arabidopsis thaliana*. *BMC Plant Biol* **9**, 32.