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NTR/NRX Define a New Thioredoxin System in the Nucleus of *Arabidopsis thaliana* Cells

Corinne Marchal[a], Valérie Delorme-Hinoux[a], Laetitia Bariat[a], Wafi Siala[a,b], Christophe Belin[a], Julio Saez-Vasquez[a], Christophe Riondet[a], and **Jean-Philippe Reichheld[a,1]**

a Université Perpignan Via Domitia, CNRS, Laboratoire Génome et Développement des Plantes, UMR 5096, 58 Avenue Paul Alduy—Bat T, F-66860, Perpignan, France
b Present address: Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium

ABSTRACT Thioredoxins (TRX) are key components of cellular redox balance, regulating many target proteins through thiol/disulfide exchange reactions. In higher plants, TRX constitute a complex multigenic family whose members have been found in almost all cellular compartments. Although chloroplastic and cytosolic TRX systems have been largely studied, the presence of a nuclear TRX system has been elusive for a long time. Nucleoredoxins (NRX) are potential nuclear TRX found in most eukaryotic organisms. In contrast to mammals, which harbor a unique NRX, angiosperms generally possess multiple NRX organized in three subfamilies. Here, we show that *Arabidopsis thaliana* has two *NRX* genes (*AtNRX1* and *AtNRX2*), respectively, belonging to subgroups I and III. While NRX1 harbors typical TRX active sites (WCG/PPC), NRX2 has atypical active sites (WCRPC and WCPPF). Nevertheless, both NRX1 and NRX2 have disulfide reduction capacities, although NRX1 alone can be reduced by the thioredoxin reductase NTRA. We also show that both NRX1 and NRX2 have a dual nuclear/cytosolic localization. Interestingly, we found that NTRA, previously identified as a cytosolic protein, is also partially localized in the nucleus, suggesting that a complete TRX system is functional in the nucleus. We show that NRX1 is mainly found as a dimer *in vivo*. *nrx1* and *nrx2* knockout mutant plants exhibit no phenotypic perturbations under standard growth conditions. However, the *nrx1* mutant shows a reduced pollen fertility phenotype, suggesting a specific role of NRX1 at the haploid phase.

Key words: thioredoxin; nucleoredoxin; nucleus; *Arabidopsis*.

INTRODUCTION

Dithiol-disulfide redox modification is a ubiquitous posttranslational modification regulating the structure and the activity of numerous proteins. Thioredoxins (TRX), Glutaredoxins (GRX), and Protein Disulfide Isomerases (PDI) are involved in the reduction of disulfide bridges (Meyer et al., 2009). TRX, GRX, and PDI belong to the thioredoxin superfamily of proteins, all of which share a similar tridimensional structure and overlapping biochemical functions. These proteins have a common structure called 'thioredoxin fold' which consists of a stack of β-sheets surrounded by α-helices (Holmgren, 1989). The canonical TRX active site (WCG/PPC), located at the surface of the protein, is composed of a dithiol essential for the disulfide reduction mechanism. The surrounding amino acids (Gly/Pro, Pro) are required to determine the reducing capacities of the thioredoxins and to maintain the conformation of the active site (Holmgren, 1985; Eklund et al., 1991; Roos et al., 2007). Moreover, the Trp residue preceding the catalytic Cys is also important for the stability of the TRX (Roos et al., 2010). Other residues have been demonstrated to contribute to TRX activity, mainly by maintaining the pKa of the active site. Among them are Asp27, Lys57 (*Escherichia coli* numeration) and an amino acid (generally Ile) just surrounding the highly conserved *cis*-Pro residue (Dyson et al., 1997; Ren et al., 2009).

The GRX active site is generally less conserved, being composed of dicysteinic or monocysteinic residues and variable surrounding amino acids (Rouhier et al., 2008). GRX activity is rather distinct from TRX because they are mainly involved in deglutathionylation activities. Another major difference is that GRX are generally reduced by the sulfhydryl-containing tripeptide glutathione (GSH) while TRX are reduced by thioredoxin reductases. Depending on the cellular compartment, thioredoxin reductases are reduced by NAD(P)H or ferredoxin (Meyer et al., 2009).

[1] To whom correspondence should be addressed. E-mail jpr@univ-perp.fr, tel. 00 33 4 68662117

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A characteristic of photosynthetic organisms is the very large number of TRX and GRX genes encoding proteins located in different cellular compartments. Several groups of 'classical' TRX are localized in plastids (TRXm, f, x, y, z) where they regulate light-dependent carbon metabolism, antioxidant stress response, and chloroplast development (Vieira Dos Santos and Rey, 2006; Lemaire et al., 2007; Arsova et al., 2010). h and o types of TRX are located in mitochondria and the cytosol (Laloi et al., 2001; Gelhaye et al., 2004; Meng et al., 2010). Most chloroplastic TRX are reduced by light through the ferredoxin (Fdx)/thioredoxin reductase system (FTR) while the mitochondrial and cytosolic TRX are generally reduced by NAD(P)H through the NAD(P)H-dependent thioredoxin reductases (NTR) (Jacquot et al., 1994; Rivera-Madrid et al., 1995; Laloi et al., 2001). Most TRX are soluble proteins, but several members of h-type TRX have been isolated in the endomembrane and plasma membrane systems (Meng et al., 2010; Traverso et al., 2013). Some TRXh proteins were shown to predominantly localize to the nucleus in cells under oxidative stress in wheat seeds (Serrato and Cejudo, 2003). Moreover, evidence of a nuclear TRX system has recently been shown in wheat seeds (Pulido et al., 2009).

In addition to classical TRX, different types of atypical TRX are found in all cellular compartments. Among them, CxxS, TRX-lilium, TRX-like, and Clot are proteins harboring atypical active sites (Serrato et al., 2008; Chibani et al., 2012; Meyer et al., 2012). Other homologs like CDSP32, APR, NTRc, tetratricoredoxin (TDX), or nucleoredoxins (NRX) are multidomain proteins composed of at least one TRX domain fused to other distinct domains (Setya et al., 1996; Vignols et al., 2003; Serrato et al., 2004; Meyer et al., 2012). Although an increasing number of works have reported the biochemical and biological functions of some of these isoforms, including NTRc and CDSP32 (Broin et al., 2002; Perez-Ruiz et al., 2006), other members are poorly documented.

NRX were first described in mice as multidomain proteins similar to TRX and conserved between mammalians (Kurooka et al., 1997). These proteins were first described as strictly nuclear proteins but this statement was revised and the proteins were later found in the cytosol in mice (Funato et al., 2006). The mouse NRX has a disulfide reduction capacity on insulin but its reducer has not been identified. In mice, biochemical data suggest that NRX is involved in the redox regulation of the Wnt/β-catenin signaling pathway, which is essential for early development and stem cell maintenance. NRX usually interacts with Dishevelled (Dvl), an essential adaptor protein for Wnt signaling, and blocks the activation of the Wnt pathway. Oxidative stress causes dissociation of NRX from Dvl, which enables Dvl to activate the downstream Wnt signaling pathway (Funato et al., 2006, 2010; Funato and Miki, 2010). In mammals, NRX is also involved in the NF-kB pathway by negatively regulating Toll-like receptor 4 signaling via recruitment of flightless-I to myeloid differentiation primary response genes (Hayashi et al., 2010). A vital function of NRX has been demonstrated in mouse mutants in which a splice-site *nrx* mutation causes craniofacial defects in the perinatal lethal line *l11Jus13* (Boles et al., 2009). Knocking down the *NRX* gene also dramatically affects embryonic development in Xenopus (Funato et al., 2006). The mammalian NRX was further shown to physically interact with PP2A phosphatase homologs (Funato and Miki, 2007), with the ER membrane Sec63 protein and to *trans*-activate several transcription factors *in vitro* (Hirota et al., 2000; Müller et al., 2011).

Plant NRX homologs show limited homology with the mammalian NRX (Chibani et al., 2009). A maize NRX1 homolog was described as reducing disulfide bonds *in vitro* and having both cytosolic and nuclear localization in kernel cells (Laughner et al., 1998). The *Arabidopsis* NRX1 was shown to play a major role in pollen tube growth in the pistil, but not *in vitro*, suggesting that it integrates signals from the maternal tissue and further guides the pollen tube towards the ovule (Qin et al., 2009). Recently, the poplar NRX1 subfamily was proposed to be involved among the potential drivers of the salicylic acid (SA)-modulated network (Xue et al., 2013).

Here, we characterize *NRX* homolog genes in *Arabidopsis thaliana*. We showed by a phylogenetic approach that, in contrast to other vascular plants which contain three subfamilies, *A. thaliana* only has two genes that belong to subgroups I and III. We show that both recombinant NRX1 and NRX2 proteins present disulfide reduction activity *in vitro*. Interestingly, we found NRX1 to be reduced by the cytosolic NTRA thioredoxin reductase and to be partially able to complement a yeast *trx1 trx2* mutant, suggesting that NRX1 behaves like a canonical TRX homolog. By different approaches, we also provide evidence that both NRX1 and NRX2 are localized in the cytosol and in the nucleus of plant cells. We also found NTR protein to be partially located in the nuclear fractions, suggesting that NTR/NRX1 constitutes a complete nuclear TRX system in plants. We isolated knockout *nrx1* and *nrx2* mutant plants. Although both mutant plants do not show obvious phenotypic perturbations under standard growth conditions, the *nrx1* mutants exhibit a lower pollen fertility phenotype.

RESULTS

Plant NRX Constitute Three Distinct Subgroups

In order to study the distribution of NRX in the eukaryotic kingdom, we collected NRX sequences from fully sequenced organisms and constructed a phylogenetic tree based on their amino acid sequences (Figure 1). NRX are absent in prokaryotic cells and some unicellular eukaryotes, like yeast and the unicellular green algae *Ostreococcus taurii*. However, they are found as a unique copy in vertebrates in which they constitute a rather homogenous family. They contain an atypical N-terminal TRX domain exhibiting an uncharacterized CxxSAPC followed by a typical TRX domain harboring a WCGPC active site, and a PDI-like C-terminal domain (Figure 2). In green algae, a complex *NRX* gene family organization was previously discussed by Chibani et al. (2009).