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Is There a Role for Glutaredoxins and BOLAs in the Perception of the Cellular Iron Status in Plants?

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Glutaredoxins (GRXs) have at least three major identified functions. In apoforms, they exhibit oxidoreductase activity controlling notably protein glutathionylation/deglutathionylation. In holoforms, i.e., iron–sulfur (Fe–S) cluster-bridging forms, they act as maturation factors for the biogenesis of Fe–S proteins or as regulators of iron homeostasis contributing directly or indirectly to the sensing of cellular iron status and/or distribution. The latter functions seem intimately connected with the capacity of specific GRXs to form [2Fe–2S] cluster-bridging homodimeric or heterodimeric complexes with BOLA proteins. In yeast species, both proteins modulate the localization and/or activity of transcription factors regulating genes coding for proteins involved in iron uptake and intracellular sequestration in response notably to iron deficiency. Whereas vertebrate GRX and BOLA isoforms may display similar functions, the involved partner proteins are different. We perform here a critical evaluation of the results supporting the implication of both protein families in similar signaling pathways in plants and provide ideas and experimental strategies to delineate further their functions.

Keywords: BOLA, glutaredoxins, iron–sulfur center, maturation factor, iron homeostasis

GENERAL INTRODUCTION

Many cellular reactions and biological processes require metalloproteins, among which those containing iron (Fe) cofactors such as mononuclear and dinuclear (non-heme) Fe centers, hemes and iron–sulfur (Fe–S) clusters, are particularly crucial. Unlike other metals such as copper or zinc, there is no universal Fe chaperone described and so far, only poly rC-binding proteins (PCBPs) were shown to coordinate Fe entry in the cytosol and serve for the metalation of non-heme Fe enzymes in mammals (Philpott et al., 2017). In contrast, the synthesis/assembly of hemes and Fe–S clusters requires more complex and universally conserved pathways (Couturier et al., 2013; Barupala et al., 2016). The machineries dedicated to the maturation of Fe–S proteins present in mitochondria

115 and chloroplasts, named ISC (iron–sulfur cluster) and SUF
 116 (sulfur mobilization), respectively, are also found in bacteria
 117 (Lill, 2009). On the other hand, cytosolic and nuclear Fe–S
 118 proteins are matured via the eukaryote-specific cytosolic iron–
 119 sulfur cluster assembly (CIA) machinery, which is, however,
 120 dependent on the mitochondrial ISC machinery for sulfur supply
 121 (Lill, 2009). Hence, given the high cellular demand for iron,
 122 sophisticated systems exist to control Fe uptake and intracellular
 123 distribution due to its potential toxicity. Strikingly, the Fe sensing
 124 systems and associated transcription factors generally differ in
 125 bacteria, yeast/fungi, mammals, and plants, but might include
 126 common actors such as glutaredoxins (GRXs) and BOLAs
 127 (Couturier et al., 2015).

128 Two GRX classes, I and II, are present in most
 129 organisms whereas additional classes are specific to some
 130 species/genus/kingdoms (Alves et al., 2009; Couturier et al.,
 131 2009). GRXs of the first class are involved in redox regulation,
 132 reducing protein disulfides or glutathione-protein mixed
 133 disulfides. GRXs from class II participate in the regulation
 134 of Fe homeostasis (Mühlenhoff et al., 2010; Haunhorst et al.,
 135 2013) and in the maturation of Fe–S proteins owing to
 136 their capacity to ligate and exchange [2Fe–2S] clusters with
 137 partner proteins (Table 1; Rodríguez-Manzaneque et al., 2002;
 138 Bandyopadhyay et al., 2008). They are also referred to as
 139 monothiol GRXs or CGFS GRXs owing to their conserved CGFS
 140 active site signature.

141 Regarding the BOLA family, an extensive phylogenetic
 142 analysis allowed delineating four groups, namely BOLA1–
 143 BOLA4 (Couturier et al., 2014). BOLA1s are present in both
 144 bacteria and eukaryotes, BOLA2s and BOLA3s in eukaryotes
 145 and BOLA4s in photosynthetic organisms, archaea, and bacteria.
 146 Pioneer works revealed functions for *Escherichia coli* BOLA in
 147 the regulation of cell morphology, possibly as a transcriptional
 148 regulator (Aldea et al., 1989), for *Saccharomyces cerevisiae*
 149 cytosolic Bol2/Fra2 (Fe repressor of activation 2) in the regulation
 150 of iron homeostasis (Lesuisse et al., 2005; Kumánovics et al.,
 151 2008), and for mitochondrial BOLAs (human BOLA3 and yeast
 152 Bol1, Bol3) in the maturation of Fe–S clusters (Table 1; Cameron
 153 et al., 2011; Melber et al., 2016; Uzarska et al., 2016).

154 A very close relationship between class II GRXs and
 155 BOLAs was initially evident from genome (gene co-occurrence
 156 and clustering, existence of fusion proteins) and large-scale
 157 interactomic analyses in various organisms (reviewed in
 158 Przybyla-Toscano et al., 2017). Then, the molecular and
 159 structural determinants of the complexes were investigated in
 160 detail using mutational, spectroscopic and structural analyses
 161 on recombinant proteins. This led to demonstrate that class
 162 II GRXs and BOLAs form both apo- and holo-heterodimers
 163 bridging a [2Fe–2S] cluster, usually more stable than the [2Fe–
 164 2S] cluster-bridging GRX homodimers, and to identify the
 165 residues serving as ligands (Li and Outten, 2012; Couturier
 166 et al., 2015; Przybyla-Toscano et al., 2017). In GRX-BOLA holo-
 167 heterodimers, the [2Fe–2S] cluster is ligated using the GRX
 168 conserved cysteine, a cysteine from glutathione (as in GRX holo-
 169 homodimers), and, on the BOLA side, using a C-terminally
 170 located conserved histidine and an histidine or a cysteine
 171 in the β 1– β 2 loop, referred to as [H/C] loop (Figure 1A;

Li et al., 2011, 2012; Roret et al., 2014; Dlouhy et al., 2016; 172
 Nasta et al., 2017). 173

174 Hereafter, based on the most recent results and known roles
 175 in non-photosynthetic organisms, we discuss the putative or
 176 confirmed functions of GRX and BOLA, alone or in complex, in
 177 photosynthetic organisms. 178
 179

180 THE CLASS II GRX AND BOLA COUPLE 181 PROTEINS PRESENT IN BACTERIA OR 182 IN EUKARYOTE ORGANELLES ARE 183 INVOLVED IN THE SYNTHESIS OF FE–S 184 CLUSTERS 185 186

187 The first evidence about GRX involvement in the biogenesis
 188 of Fe–S proteins were obtained from a *S. cerevisiae* mutant
 189 for the mitochondrial Grx5 (Table 1; Rodríguez-Manzaneque
 190 et al., 2002; Mühlenhoff et al., 2003). Orthologs of this single
 191 domain-containing GRX are found in bacteria, archaea and
 192 plant plastids. The current view is that Grx5 is required for
 193 the maturation of all types of Fe–S clusters in mitochondria,
 194 receiving a [2Fe–2S] cluster from ISCU-type scaffold proteins
 195 and transferring it to ISCA-type transfer proteins for subsequent
 196 maturation of [4Fe–4S] proteins (Figure 1B). Grx5 is also
 197 required for the maturation of nucleo-cytosolic Fe–S proteins and
 198 the activation of the Aft1 transcription factor, pointing to its key
 199 position in *S. cerevisiae* (see below) (Uzarska et al., 2013). Yeast
 200 Bol1 and Bol3, which have the capacity to form heterodimers
 201 with Grx5, were later shown to be required for a specific set
 202 of mitochondrial [4Fe–4S] proteins, without affecting *de novo*
 203 synthesis of [2Fe–2S] proteins (Uzarska et al., 2016). So far,
 204 human BOLA3, but not BOLA1, was demonstrated as required
 205 for the maturation of specific Fe–S proteins (Table 1; Cameron
 206 et al., 2011; Willems et al., 2013). The client proteins are notably
 207 the succinate dehydrogenase/complex II and lipoate synthase.
 208 Moreover, the fact that *bol1-bol3* Δ mutants are neither affected
 209 in the CIA machinery, nor in Aft1 activation, indicates that Grx5
 210 has physiological roles independent of Bol1 and Bol3 (Uzarska
 211 et al., 2016). Additional studies suggested that Bol1 indeed
 212 acts early in the ISC pathway in concert with Grx5 (possibly
 213 only for [4Fe–4S] proteins) whereas Bol3 may preferentially act
 214 with NFU1, a late Fe–S cluster transfer protein, to preserve
 215 the [4Fe–4S] center found in some specific mitochondrial client
 216 proteins, as lipoate synthase, from oxidative damage (Figure 1B;
 217 Melber et al., 2016).

218 Concerning bacteria, the sole Grx isoform, Grx4/D, and both
 219 BolAs (BolA and IbaG) from *E. coli* were recently shown as
 220 implicated in the maturation of the respiratory complexes I
 221 and II, but the effects are only visible when multiple genes
 222 are mutated (Burschel et al., 2019). This role in maturing
 223 Fe–S proteins is consistent with (i) the synthetic lethality
 224 of the *Grx4* gene with genes present in the ISC operon
 225 (Butland et al., 2008), (ii) the interaction *in vitro* of Grx4
 226 with the MiaB Fe–S protein (Boutigny et al., 2013), and
 227 (iii) the capacity of Grx4-BolA and Grx4-IbaG to form the
 228 usual [2Fe–2S] cluster-bridging heterodimers (Yeung et al., 2011;

	Organism	Protein names	Mutant phenotype(s)	References			
229	TABLE 1 Iron-related phenotypes of <i>bolA</i> and <i>glutaredoxin</i> mutants from various sources.					286	
230					287		
231					288		
232	Mono-domain (organellar) GRXs	<i>Saccharomyces cerevisiae</i>	Grx5	Defaults in Fe–S cluster assembly	Rodríguez-Manzanera et al., 2002; Mühlhoff et al., 2003	289	
233		<i>Schizosaccharomyces pombe</i>	Grx5	Defaults in Fe–S cluster assembly, decreased amount of mitochondrial DNA, reduced growth, and sensitivity toward oxidants	Chung et al., 2005; Kim et al., 2010	290	
234		<i>Danio rerio</i>	GRX5	Embryo lethal	Wingert et al., 2005	291	
235		<i>Homo sapiens</i>	GLRX5	Defaults in Fe–S cluster assembly leading to sideroblastic anemia	Camaschella et al., 2007; Ye et al., 2010	292	
236		<i>Trypanosoma brucei</i>	1-C-Grx1	Lethal	Comini et al., 2008	293	
237		<i>Sinorhizobium meliloti</i>	Grx2	Defaults in Fe–S cluster assembly, deregulation of RirA transcriptional activity, increased intracellular iron content, modified nodule development	Benyamina et al., 2013	294	
238		<i>Escherichia coli</i>	Grx4	Sensitivity to iron depletion, defect in respiratory complex I	Yeung et al., 2011; Burschel et al., 2019	295	
239		<i>Arabidopsis thaliana</i>	GRXS14	Sensitivity to prolonged darkness	Rey et al., 2017	296	
240		<i>Arabidopsis thaliana</i>	GRXS15	Lethal, decreased amounts of lipoate synthase and of lipoic acid dependent H subunits of the glycine cleavage system in RNAi lines	Moseler et al., 2015; Ströher et al., 2016	297	
241		<i>Arabidopsis thaliana</i>	GRXS16	None described for co-suppressed and RNAi lines	Rey et al., 2017	298	
242		Multi-domain (cytosolic) GRXs	<i>Saccharomyces cerevisiae</i>	Grx3	Impaired regulation of Aft1/2 and iron homeostasis	Ojeda et al., 2006; Pujol-Carrion et al., 2006	299
243			<i>Saccharomyces cerevisiae</i>	Grx4	Impaired regulation of Aft1/2 and iron homeostasis	Ojeda et al., 2006; Pujol-Carrion et al., 2006	300
244	<i>Saccharomyces cerevisiae</i>		Grx3–Grx4	Lethal in some background. Impaired iron trafficking and assembly of Fe–S proteins, heme, and iron-containing proteins	Pujol-Carrion et al., 2006; Mühlhoff et al., 2010	301	
245	<i>Schizosaccharomyces pombe</i>		Grx4	Lethal	Chung et al., 2005	302	
246	<i>Cryptococcus neoformans</i>		Grx4	Slow growth upon iron deprivation	Attarian et al., 2018	303	
247	<i>Danio rerio</i>		GRX3	Impaired heme synthesis and Fe–S protein maturation	Haunhorst et al., 2013	304	
248	<i>Homo sapiens</i>		GLRX3/PICOT	Decreased activities of cytosolic Fe–S proteins	Haunhorst et al., 2013	305	
249	<i>Arabidopsis thaliana</i>		GRXS17	Growth defects (meristem arrest) upon elevated temperature and long photoperiod. No decrease in cytosolic Fe–S protein activity	Cheng et al., 2011; Knuesting et al., 2015; Yu et al., 2017	306	
250	BOLA		<i>Saccharomyces cerevisiae</i>	Bol1	No growth defect and no decrease in Fe–S enzyme activity	Melber et al., 2016; Uzarska et al., 2016	307
251			<i>Saccharomyces cerevisiae</i>	Bol3	Slightly decreased complex II (SDH) activity	Melber et al., 2016; Uzarska et al., 2016	308
252			<i>Saccharomyces cerevisiae</i>	Bol1–Bol3	Decreased activity of lipoic acid-dependent enzymes, aconitase, and respiratory complex II	Melber et al., 2016; Uzarska et al., 2016	309
253			<i>Saccharomyces cerevisiae</i>	Bol2/Fra2	Impaired regulation of Aft1/2 and iron homeostasis	Kumánovics et al., 2008; Uzarska et al., 2016	310
254		<i>Schizosaccharomyces pombe</i>	BolA2/ Fra2	Impaired regulation of the Fep1 transcription factor	Jacques et al., 2014	311	
255		<i>Homo sapiens</i>	BOLA1	Oxidation of the mitochondrial GSH pool	Willems et al., 2013	312	
256	<i>Homo sapiens</i>	BOLA2	None described for siRNA lines	Frey et al., 2016	313		
257	<i>Homo sapiens</i>	BOLA3	Defect in lipoic acid-dependent enzymes and in respiratory complexes I and II	Cameron et al., 2011	314		
258	<i>Escherichia coli</i>	BolA	Partial defect in respiratory complex I assembly	Burschel et al., 2019	315		
259	<i>Escherichia coli</i>	IbaG	None described	Burschel et al., 2019	316		
260	<i>Escherichia coli</i>	BolA – IbaG	Decreased complex II activity	Burschel et al., 2019	317		
261	<i>Salmonella typhimurium</i>	BolA	Decreased resistance to acidic and oxidative stresses and decreased virulence	Mil-Homens et al., 2018	318		
262	<i>Arabidopsis thaliana</i>	BOLA2	None described under control conditions, increased resistance to oxidative conditions	Qin et al., 2015	319		
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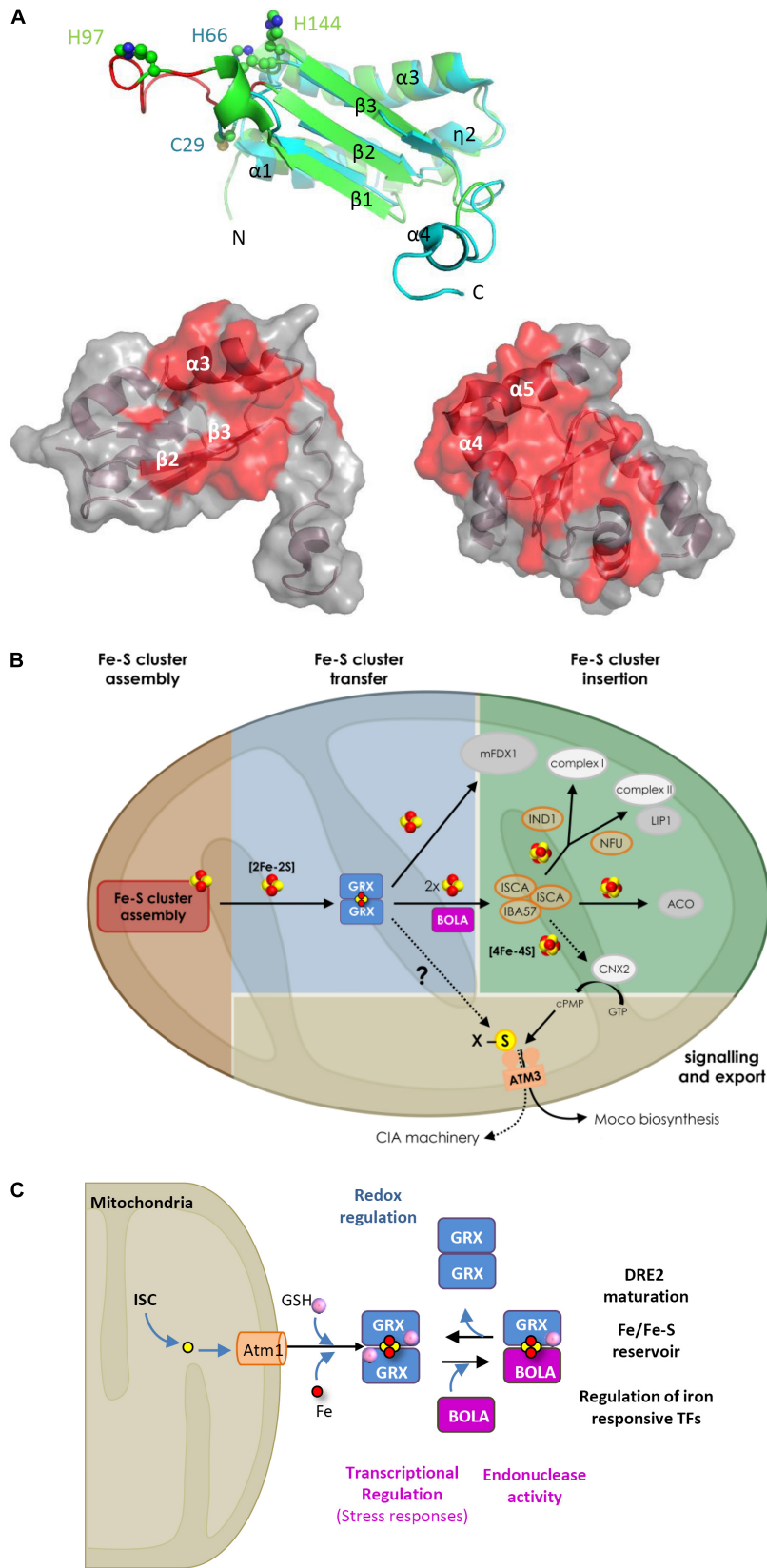


FIGURE 1 | Continued

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FIGURE 1 | Properties and hypothetical roles of the class II GRX-BOLA couple in plants. **(A)** Tridimensional structures of plastidial *Arabidopsis thaliana* BOLA1 and GRXS14 proteins highlighting the residues involved in the interactions. On the top, superimposition of AtBOLA1 (green) and AtBOLA2 (blue) structures. Both proteins have a α/β -structure made of four helices and three strands with an $\alpha 1\beta 1\beta 2\eta 2\alpha 3\beta 3\alpha 4$ (η : 3_{10} -helix) topology (Roret et al., 2014). The β -strands form a central three-stranded β -sheet. In addition to the extended C-terminal part in AtBOLA2, both proteins differ by the length of the $\beta 1$ – $\beta 2$ loop (in red in BOLA1), referred to as [H/C] loop, and which contains the histidine (His97 in AtBOLA1) and cysteine (Cys29 in AtBOLA2) residues provided by BOLA proteins for Fe–S cluster bridging together His66 (AtBOLA2) or His144 (AtBOLA1). The putative DNA binding site in BOLAs is formed by the $\eta 2$ and $\alpha 3$ helices, the loop containing a specific FXGX signature (type II β -turn) and the $\alpha 3$ helix containing a positively charged RHR motif and the $\beta 3$ strand. Below, from left to right, AtBOLA2 structure showing 18 residues (mainly part of the $\beta 2$ and $\beta 3$ strands and $\alpha 3$ helix) identified by NMR titration as involved in the interaction with apo-AtGRXS14; and AtGRXS14 structure showing 32 residues (many present in the C-terminal $\alpha 3$ and $\alpha 4$ helices) identified by NMR titration as involved in the interaction with AtBOLA2 (Roret et al., 2014). These residues, plus some additional ones, are also involved in the formation of the [2Fe–2S] cluster-bridged heterodimer as determined using human proteins (Nasta et al., 2017). **(B)** Hypothetical model for the role of GRXS15 and BOLA4 in plant mitochondria. By analogy with the yeast system, GRXS15 (shortened as GRX) should receive a [2Fe–2S] cluster synthesized *de novo* by a multi-protein assembly complex (details about the proteins involved in the early steps of Fe–S cluster assembly and transfer have been omitted). GRXS15 is supposed to transfer its [2Fe–2S] cluster to client proteins as the mitochondrial ferredoxin 1 (mFDX1) (Moseler et al., 2015) or to ISCA proteins for the reductive conversion of two [2Fe–2S] clusters into a [4Fe–4S] cluster (as shown with human proteins) and its subsequent delivery to client proteins bearing such cluster. In the absence of genetic analysis about *bola4* mutants, the contribution of BOLA4 (shortened as BOLA) for the respective roles of GRXS15 is unclear, but the confirmed interaction between both proteins (Couturier et al., 2014) prompted us to include BOLA at this step as yeast Bol1/3 proteins are only required for the maturation of [4Fe–4S] proteins. The specific defects observed for aconitase (ACO) and lipoic-acid dependent proteins in the *GRXS15* mutant lines indicate a direct or indirect role for GRXS15 in the maturation of both lipoate synthase (LIP1) and aconitase. Finally, whether GRXS15 is required for the maturation and activity of cytosolic and nuclear Fe–S proteins by fueling the CIA machinery as shown for yeast Grx5, or by indirectly contributing to the synthesis of molybdenum cofactor, that is present in several cytosolic Fe–S proteins, is unknown. **(C)** Roles associated with the various oligomeric forms involving nucleocytosolic GRXs and BOLAs irrespective of the organisms considered. The color code is as follows: in blue, functions associated with apo-dimeric GRX forms, in purple those associated with apo-BOLA and in black those associated with the GRX homodimeric or GRX-BOLA heterodimeric forms bridging a [2Fe–2S].

Dlouhy et al., 2016). In *Sinorhizobium meliloti*, deletion of the sole class II GRX also leads to impaired maturation of Fe–S proteins and increased intracellular iron content (Benyamina et al., 2013).

In plants, the corresponding mitochondrial GRX is named GRXS15. Knockout *Arabidopsis* mutants are lethal due to defective embryo development (Moseler et al., 2015). Plants expressing a mutated GRXS15 form modified for its ability to coordinate an Fe–S cluster exhibit severely reduced growth and impaired aconitase activity (Moseler et al., 2015). Additionally, *Arabidopsis* GRXS15 down-regulated lines display slowed growth and impaired activity of enzymes dependent on lipoic acid, the synthesis of which is ensured by the Fe–S cluster-containing lipoyl synthase (Ströher et al., 2016). Whether GRXS15 fulfills its function in concert with BOLA4, the sole mitochondrial BOLA, remains to be explored, but their interaction was demonstrated in yeast and *in planta* (Figure 1B; Couturier et al., 2014). Plants also have class II GRXs (GRXS14 and S16) and mono-domain BOLAs (BOLA1, BOLA4) in plastids (Couturier et al., 2013). So far, *in planta* evidence for their implication in the biogenesis of Fe–S proteins are scarce (Table 1). GRXS14-deficient *Arabidopsis* plants exhibit accelerated chlorophyll loss upon prolonged darkness, a treatment also leading to a decreased abundance of proteins acting in Fe–S cluster metabolism (Rey et al., 2017). Nevertheless, the demonstration that *Arabidopsis* and/or poplar GRXS14 and GRXS16 interact both with BOLA1 and BOLA4 (Couturier et al., 2014), bind Fe–S clusters alone or in complex (Bandyopadhyay et al., 2008; Dhalleine et al., 2014; Roret et al., 2014) and can transfer it to partner proteins (Mapolelo et al., 2013) give strong credence to such a role. Even more importantly, all plant GRX and BOLA genes complement totally or partially (*GRXS15*) the corresponding yeast *grx5* and *bol1-bol3* mutants, indicating that they possess similar structural and functional determinants (Bandyopadhyay et al., 2008; Moseler et al., 2015; Uzarska et al., 2018).

MULTIPLE FUNCTIONS IN THE REGULATION OF IRON HOMEOSTASIS OF THE CLASS II GRX AND BOLA COUPLE IN THE CYTOSOL/NUCLEUS OF EUKARYOTES

Eukaryote cytosolic class II GRXs are multidomain proteins formed by an N-terminal thioredoxin-like domain fused to one to three GRX domains (Couturier et al., 2009). Most organisms have a single GRX of this type and also a single cytosolic BOLA isoform, referred to as BOLA2/Bol2/Fra2. The pioneering studies showing the involvement of class II GRXs and BOLAs in Fe homeostasis have been conducted in *S. cerevisiae* mutants deregulated in *Grx3*, *Grx4*, and *Bol2/Fra2* genes (Table 1; Lesuisse et al., 2005; Ojeda et al., 2006; Pujol-Carrion et al., 2006). In yeast, the regulation of Fe concentration is achieved at the transcriptional level by low-(Aft1 and Aft2) and high-level (Yap5) sensing transcription factors and at the post-transcriptional level by mRNA-binding proteins (Outten and Albetel, 2013). Both types of transcription factors bind [2Fe–2S] clusters allowing them to perceive the cellular Fe or Fe–S cluster status (Poor et al., 2014; Rietzschel et al., 2015). Whereas *Grx4* expression is regulated by Yap5, it is not documented whether Yap5 localization or activity is controlled by a GRX/BOLA complex. Regarding Aft1/Aft2, their subcellular (nuclear vs. cytosolic) localization is controlled by a Fra2-Grx3/4 inhibitory complex (possibly requiring also the aminopeptidase Fra1) (Kumánovics et al., 2008). The current view is that the presence of an Fe–S cluster in the Fra2-Grx3/4 complex is synonymous of iron-replete conditions and of a correct functioning of the ISC machinery (Figure 1C; Kumánovics et al., 2008). By transferring clusters to Aft1/2, the GRX-BOLA complex should either retain them in the cytosol or promote their dissociation from DNA if in the nucleus (Ueta et al., 2012; Poor et al., 2014).

571 Some aspects of Fe homeostasis in other yeasts and fungi
572 are also controlled by GRX and/or BOLA. In *Cryptococcus*
573 *neoformans*, Fe depletion promotes Grx4 relocation from the
574 nucleus to the cytoplasm allowing the regulation of Cir1, a
575 master regulator of Fe-responsive genes (Attarian et al., 2018).
576 In *Schizosaccharomyces pombe*, Fe metabolism is regulated by
577 two transcriptional repressors, the GATA-type iron sensing Fep1
578 and the CCAAT-binding factor complex subunit Php4 (Brault
579 et al., 2015). Their localization and/or DNA binding activity
580 are regulated by Grx4 and/or Fra2 (reviewed in Outten and
581 Albetel, 2013; Brault et al., 2015). The binding of a [2Fe–2S]
582 cluster between Grx4 and Php4 may promote Php4 release
583 from the CCAAT-binding complex at the DNA targets and
584 suppress its inhibitory effect on the expression of Fe storage
585 genes (Dlouhy et al., 2017). Unlike Php4, the regulation of which
586 does not involve Fra2, the formation of a [2Fe–2S]-Grx4/Fra2
587 heterodimeric complex is required for regulating Fep1 activation
588 (Jacques et al., 2014; Encinar del Dedo et al., 2015).

589 In mammals, the regulation of Fe metabolism and homeostasis
590 is ensured by IRP1/2 and RNA-binding proteins (Rouault and
591 Maio, 2017). Under Fe limitation, both IRPs bind to the so-
592 called Iron Responsive Elements (IREs) in untranslated regions
593 of mRNAs coding for proteins implicated in Fe assimilation and
594 homeostasis (Rouault and Maio, 2017). Doing so, they control
595 either mRNA stabilization or translational blocking. Whereas
596 IRP2 release from IREs is mediated by proteasomal degradation
597 (Guo et al., 1995), IRP1 function may depend on GLRX3/PICOT
598 (but also on mitochondrial GLRX5) as it relies on the binding
599 of an Fe–S cluster. Under Fe sufficiency, IRP1 binds a [4Fe–4S]
600 cluster and acts as an aconitase whereas under Fe limitation the
601 protein turns into an apoform binding to IREs. Consequently,
602 IRP1 requires functional mitochondrial and cytosolic Fe–S
603 cluster assembly machineries. Having two GRX domains, human
604 GLRX3 forms homodimers or heterotrimers with two BOLA2
605 molecules bridging two [2Fe–2S] clusters (Li et al., 2012; Banci
606 et al., 2015b; Frey et al., 2016). It also binds a [4Fe–4S] cluster
607 and transfers it *in vitro* to an apo-IRP1 (Xia et al., 2015).
608 *GLRX3* silencing in human HELA cells decreased the activity
609 of several cytosolic Fe–S proteins, including IRP1 (Table 1;
610 Haunhorst et al., 2013). In zebrafish, *GLRX3* deletion impaired
611 heme biosynthesis during embryo development (Haunhorst et al.,
612 2013). All of this indicates important functions of vertebrate
613 *GLRX3* in Fe metabolism.

614 In addition to an Fe sensing function, an Fe or Fe–S cluster
615 trafficking function was proposed for yeast Grx3/4 and the
616 human GLRX3-BOLA2 complex to ensure proper assembly of
617 several types of Fe-containing centers. In fact, most multidomain
618 GRXs are able to rescue the Fe–S cluster maturation defects
619 of the yeast *grx5* mutant (Molina et al., 2004; Bandyopadhyay
620 et al., 2008; Knesting et al., 2015) suggesting that they have the
621 capacity of exchanging Fe–S clusters. Accordingly, both human
622 *GLRX3* homodimers and *GLRX3*-BOLA2 trimeric complexes
623 bridging two [2Fe–2S] clusters can deliver their clusters to the
624 anamorsin/CIAPIN/DRE2 protein (Banci et al., 2015a,b). From
625 the observation that the maturation of yeast Grx3/4 and human
626 *GLRX3*-BOLA2 heterodimers require the mitochondrial ISC
627 machinery but not CIA components (Mühlenhoff et al., 2010;

Frey et al., 2016), it is concluded that cytosolic class II GRXs
628 should build their cluster from a sulfur compound exported
629 by the mitochondrial ATM transporter (Figure 1C). In yeast
630 *grx3/4Δ*, the Fe or Fe-cofactor insertion in various proteins
631 present in cytosol [catalase, ribonucleotide reductase (RNR)],
632 and mitochondria (complexes II and III, aconitase, Coq7 mono-
633 oxygenase) is altered (Mühlenhoff et al., 2010; Zhang et al.,
634 2011). Moreover, the respective increased and decreased Fe
635 levels in cytosol and mitochondria of Grx3/4 depleted cells
636 pointed to impaired Fe distribution (Mühlenhoff et al., 2010).
637 These additional functions of yeast Grx3/4 are well exemplified
638 in the case of RNR di-iron cofactor biogenesis because Grx4
639 provides the Fe atoms, but also serves for the maturation of
640 holo-Dre2, that provides the required electrons (Li et al., 2017).
641 A contribution of yeast Bol2 for these functions is unclear
642 even though a general role in cytosolic Fe/S protein maturation
643 is excluded (Uzarska et al., 2016). In Human, GLRX3-BOLA2
644 trimeric complexes bridging two [2Fe–2S] clusters were proposed
645 to constitute a reservoir for delivering Fe or Fe–S cluster to
646 some Fe-containing target proteins based notably on the six-
647 eightfold increased abundance observed in response to elevated
648 iron (Frey et al., 2016).

649 The function of GRXS17, the sole nucleo-cytosolic class II
650 GRX in plants, has been explored using several approaches.
651 Tandem affinity purification using a tagged GRX form expressed
652 in Arabidopsis cell cultures and seedlings pointed to the
653 association of GRXS17 with CIA components and BOLA2 (Iñigo
654 et al., 2016). The interactions with DRE2 and BOLA have been
655 confirmed *in vivo* by binary yeast two-hybrid and BiFC and/or
656 *in vitro* by co-expression in *E. coli* (Couturier et al., 2014;
657 Dhalleine et al., 2014; Iñigo et al., 2016). As GRXs interact with
658 Dre2/Anamorsin in yeast and human cells (Zhang et al., 2011;
659 Banci et al., 2015b), the only direct CIA partner of GRXS17 might
660 be DRE2 and the other proteins part of a complex. Besides, the
661 binding of GRXS17 with putative Fe–S client proteins involved in
662 purine salvage (xanthine dehydrogenase 1) or tRNA modification
663 (thiouridylase subunits 1 and 2) was shown (Iñigo et al.,
664 2016). Thus, one would expect that plants deficient in GRXS17
665 display a marked phenotype in relation with Fe metabolism,
666 but the analysis of Arabidopsis *grxs17* plants led to relatively
667 complex data. Indeed, their development is only mildly affected
668 under standard growth conditions, but gets severely impaired
669 (elongated leaves, modified shoot apical meristem structure,
670 and altered auxin response) at high temperature or under long
671 photoperiod (Cheng et al., 2011; Knesting et al., 2015). It is
672 not yet clear whether a redox- and/or an Fe-related function
673 of GRXS17 is responsible for these alterations. In fact, *in vitro*
674 pull-downs performed using the recombinant protein allowed
675 recovering many non-Fe–S proteins including the NF-YC11
676 transcriptional regulator (Knesting et al., 2015). Moreover, there
677 is no variation in the Fe content in mature leaves and only
678 a slight increase in seeds (Yu et al., 2017) and *grxs17* plants
679 exhibit no or minor decreases in the activity of three Fe–S
680 containing enzymes: aconitase, aldehyde oxidase and xanthine
681 dehydrogenase (Knesting et al., 2015; Iñigo et al., 2016). On the
682 other hand, GRXS17-deficient lines exhibit a slightly increased
683 sensitivity to genotoxic stress which is reminiscent of mutants
684

685 compromised in the CIA pathway (Iñigo et al., 2016). Finally,
 686 when GRXS17-deficient lines are exposed to Fe deficiency, the
 687 primary root growth reduction is exacerbated and ROS levels
 688 are elevated (Iñigo et al., 2016; Yu et al., 2017). Whether
 689 plant GRXS17 and BOLA2 act in concert remains unclear. The
 690 Arabidopsis *bola2* (incorrectly named *bola3*) mutant displays no
 691 phenotype under control conditions and no change in the activity
 692 of typical Fe-S enzymes (Qin et al., 2015). Surprisingly, this
 693 line is more tolerant to oxidative stress generated by Fe excess
 694 (Qin et al., 2015). In conclusion, *bola2* and *grxs17* plants exhibit
 695 relatively mild phenotypes, visible mostly under stress conditions,
 696 compared to those described for human and yeast orthologs and
 697 to the embryo-lethality of most Arabidopsis mutants defective for
 698 early acting CIA components (Bernard et al., 2013). This raises
 699 some questions about the exact functions of BOLA2 and GRXS17
 700 in the regulation of Fe homeostasis in plant cells and about the
 701 existence of an alternative system, notably for delivering Fe-S
 702 clusters to DRE2, whose function is essential.

704 ROADMAP TOWARD THE 705 UNDERSTANDING OF THE ROLES OF 706 GRX/BOLA COUPLES IN PLANTS 707

708 **Q9** In this section, we propose some ideas and experimental
 709 strategies that should warrant deciphering the functions
 710 associated to GRX/BOLA couples in plants.

711 Evidence obtained so far indicate that the class II mono-
 712 domain GRXs and BOLAs present in mitochondria of non-
 713 plant eukaryotes and in bacteria act as maturation factors for
 714 the biogenesis of Fe-S proteins. A similar role seems true for
 715 the plant mitochondrial GRXS15, but it is now mandatory to
 716 examine whether it also contributes to the maturation of extra-
 717 mitochondrial proteins in plants. Another challenge will be to
 718 understand why it is essential in plants unlike in yeast. Also,
 719 the physiological consequences of *BOLA4* depletion must be
 720 investigated to see whether this fits with a function connected
 721 to GRXS15. Concerning plastidial proteins (GRXS14, GRXS16,
 722 BOLA1, and BOLA4), a role in the maturation of Fe-S proteins
 723 still needs to be demonstrated *in planta*, despite they can
 724 functionally substitute to their mitochondrial yeast counterparts.

725 With regard to the cytosolic multi-domain GRXs and BOLAs,
 726 a role in Fe metabolism seems evolutionary conserved, but their
 727 contribution and partners differ. In yeast, their primary function
 728 is to regulate Fe-responsive transcription factors. Additional
 729 functions are to ensure a proper Fe distribution toward all types
 730 of Fe cofactors (including heme and non-heme Fe centers) and/or
 731 to serve for Dre2 maturation, thus contributing to the correct
 732 functioning of the CIA machinery. In this case, Grx3/4 have
 733 an exclusive or predominant role because the corresponding
 734 mutant is lethal or strongly affected, unlike the *bol2/fra2* mutant.
 735 Experimental evidence indicate that the involvement of GRX
 736 and/or BOLA in DRE2 maturation is likely also true in mammals
 737 and plants, but evidence supporting other functions are scarce.

738 A first prerequisite to future molecular and physiological
 739 analyses is to generate the missing single knock-out lines but
 740 also multiple knock-out lines for possibly redundant proteins.

741 This would be particularly important to obtain lines combining
 742 mutations for GRXS14 and GRXS16, for BOLA1 and BOLA4,
 743 but also for GRXS17 and the only other Fe-S ligating GRXs
 744 reported so far in the cytosol, namely GRXC1 (Rouhier et al.,
 745 2007), or BOLA2. In case the single or multiple mutants are lethal,
 746 an option for obtaining viable lines would be to generate RNAi
 747 lines as for *GRXS15*, but also dominant negative mutant lines
 748 expressing mutated versions of GRX or BOLA unable for instance
 749 to ligate the Fe-S cluster, i.e., mutated for the catalytic cysteine of
 750 GRXs or the conserved histidine residue of BOLA.

751 At the physiological level, the growth of these plants
 752 should be analyzed under standard conditions, but also under
 753 environmental constraints as for instance the phenotypes of
 754 *grxs17* mutants are only visible in specific conditions. For
 755 the BOLA2-GRXS17 couple, understanding their connection
 756 and discriminating between Fe- or redox-related functions
 757 will require in particular to assess the phenotypes of the
 758 corresponding mutants in the same experimental setup and
 759 conditions. Considering the described importance of GSH
 760 for ligating Fe-S cluster in GRX homodimer or GRX-
 761 BOLA heterodimer and for the maturation of cytosolic Fe-S
 762 proteins (Sipos et al., 2002), crossing some of these mutants
 763 with mutants having an altered GSH homeostasis would
 764 certainly be informative.

765 In other respects, an obvious strategy is to measure the
 766 abundance/activity of representative Fe-S proteins in these lines.
 767 However, performing quantitative proteomic and metabolomic
 768 approaches may be more informative and help obtaining a
 769 broader view of the molecular and cellular mechanisms affected
 770 and of the compensations established. It may also rapidly point
 771 to metabolic differences existing among mutants.

772 In all cases, determining the identity of the direct and indirect
 773 targets of both GRXs and BOLAs would represent a mandatory
 774 information. For instance, the proteins involved in the Fe-S
 775 cluster maturation process may act at different steps. Various
 776 approaches complementary to quantitative proteomics proved
 777 valuable even for detecting supposedly transient interactions
 778 among Fe-S cluster donors and acceptors (Touraine et al., 2019).
 779 Hence, it is possible to combine it to another non-targeted
 780 approach such as co-immunoprecipitation or to binary yeast two
 781 hybrid experiments which has the advantage for instance to allow
 782 studying rapidly sequence requirements by mutational analysis.

783 In summary, the combination of genetic approaches, omics
 784 analyses and conventional biochemical tools should in principle
 785 allow better delineating the roles and specificities of GRX/BOLA
 786 couples in the maintenance of Fe homeostasis in plants.

787 DATA AVAILABILITY

788 No datasets were generated or analyzed for this study.

789 AUTHOR CONTRIBUTIONS

790 All authors wrote the text and approved the final version
 791 of the manuscript. **Q10**

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