INTRODUCTION
Thioredoxins (Trx) are ubiquitous small redox-active proteins that act as electron donors in various metabolic pathways, regulate enzymatic activities, and maintain the cellular environment in a reduced state (Arnér and Holmgren, 2000). Trx contains a WC\G\P\PC active site motif and reduces disulfide bonds in target proteins through thiol-disulfide exchange reactions. Oxidized Trx is recycled by the NADPH-dependent dimeric flavoprotein Trx reductase (NTR) or the chloroplastic iron-sulfur cluster proteins ferredoxin and ferredoxin–Trx reductase coupled to the photosynthetic apparatus (Jacquot et al., 2009). In NTR, reducing the enzyme undergoes a large conformational change and the redox active CXXC motif in NTR is positioned close to the reduced cofactor (FAD) in the so-called flavin reducing (FR) conformation. Then, the enzyme returns to the FR conformation and the active site WC\G\P\PC in Trx is reduced concomitant with oxidation of the NTR CXXC motif. Plant Trx play key roles in regulation of processes such as photosynthesis, flowering, immaturity, and seed germination (Buchanan and Balmer, 2005). In comparison to other organisms, plants contain a remarkable diversity of Trx classified into groups based on sequence similarity and showing different subcellular locations (Cellinova et al., 2005). The mainly cytosolic h-type Trx is reduced by NTR and is proposed to facilitate the germination process and is recycled by NADPH-dependent Trx reductase. This review presents a summary of the research conducted during the last 10 years to elucidate the structure and function of the barley seed Trx system at the molecular level combined with proteomic approaches to identify target proteins.

Keywords: thioredoxin, disulfide bond, redox regulation, NADPH-dependent thioredoxin reductase, cereal proteomics

Thioredoxin (Trx) reduces disulfide bonds and plays numerous important functions in plants. In cereal seeds, cytosolic h-type Trx facilitates the release of energy reserves during the germination process and is recycled by NADPH-dependent Trx reductase. This review presents a summary of the research conducted during the last 10 years to elucidate the structure and function of the barley seed Trx system at the molecular level combined with proteomic approaches to identify target proteins.

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Published: 21 May 2013
doi: 10.3389/fpls.2013.00151
observed differences in HvTrx protein profiles therefore seem not to be due to regulation of transcription and probably occur at the post-translational level.

Transcripts encoding both HvNTR1 and HvNTR2 were detected in embryos isolated from mature grains and increased up to 72 h after imbibition (Shahpiri et al., 2008). Transcripts were also detected for both genes in isolated aleurone layers. HvNTR2 transcript was present at similar levels in embryo and aleurone layer whereas HvNTR1 transcripts were much less abundant in the embryo than in the aleurone layer. The HvNTR2 transcript level was reduced in aleurone layers treated with GA for up to 18 h (Shahpiri et al., 2008). Quantitative PCR (Kirkensgaard, 2011) showed a slight downregulation of HvNTR1, and a more than twofold upregulation of HvNTR2 by ≥100 nM GA after 24 h. The expression level of HvNTR2 was therefore confirmed to be 10–40 times higher than HvNTR1, suggesting that HvNTR2 is the most important isof orm in aleurone layers subjected to GA.

The levels for both HvTrx transcripts were around five times higher than even the highest level of HvNTR2. Overall, the data suggest that the activity level of the NTR/Trx system in barley grain tissues is determined by transcriptional regulation of NTR genes, coupled with post-translational regulation of HvTrx protein levels. In this context it is relevant to point out that barley microarray analysis has shown that loss of dormancy leads to increased expression of HvNTR1, HvNTR2, and HvTrx1 in embryos of imbibed grains (Barrero et al., 2009; Kirkensgaard, 2011).

**STRUCTURAL AND CATALYTIC PROPERTIES**

HvTrxh1 and HvTrxh2 show 51% sequence identity and similar biophysical characteristics. The redox potentials (E°) of both proteins was determined to be ~270 mV in a fluorometric assay using Escherichia coli Trx as a reference and the pKa of the nucleophilic active site thiol (-S-H) in both HvTrxh1 and HvTrxh2 were determined to be 7.6 by iodoadcamide (IAM) alkylation kinetics (Maeda et al., 2010). Nevertheless, HvTrxh1 displays slightly higher thiol reactivity and higher affinity for the model substrate insulin, possibly due to subtle differences in the local environment surrounding the active site. The three-dimensional crystal structures of HvTrxh1 and HvTrxh2 were determined to 1.7 and 2.0 Å resolution, respectively (Maeda et al., 2008). Both proteins display the overall fold conserved among Trx from different species with a central five-stranded β-sheet surrounded by four α-helices in a βαβαβαββα topology. Comparison of the structures of HvTrxh2 determined in oxidized and partially reduced states does not suggest major redox-dependent changes in the active site area with the exception of the side chain conformations of the redox-active cysteines (Maeda et al., 2008). Dimers of HvTrxh1 are formed in the crystal lattice and the interface is stabilized by three backbone–backbone hydrogen bonds in a pattern that resembles the inter-molecular contacts observed in Trx-target complexes (see below).

The structure of HvNTR2 was solved to 2.6 Å resolution by X-ray crystallography (Kirkensgaard, 2009). As expected, this first example of a monocotyledonous plant NTR structure reveals a dimeric protein in which each monomer is composed of FAD- and NADPH-binding domains. HvNTR2 share overall similarity to the structures of AnNTR-B from Arabidopsis thaliana and other low-molecular-weight NTRs (Jacquot et al., 2009). However, the relative position of the FAD and the NADPH domains is not the same. Compared to AnNTR-B the NADPH domain in HvNTR2 is rotated by 25° and bent by a 38% closure relative to the FAD domain. The structure may thus represent an intermediate between the FO and the FR conformations.

Given that both HvTrxh1, HvTrxh2, HvNTR1, and HvNTR2-encoding genes are expressed to some extent in all grain tissues, it was relevant to determine whether the proteins could function interchangeably. This was shown to be the case, with minor variations in catalytic efficiency (Shahpiri et al., 2008). Importantly, the activity of the system was confirmed at the relatively low pH expected in the starchy endosperm of germinating grains (Shahpiri et al., 2008).

**PROTEOMIC APPROACHES FOR IDENTIFICATION OF BARLEY Trx H TARGET PROTEINS**

Target proteins of barley Trx h have been identified by different proteomic approaches applied to extracts of barley grain tissue. Briefly, protein extracts are incubated in the presence of recombinant Trx h and reduced thiols are labeled with specific reagents that are either visualized after separation by two-dimensional gel electrophoresis (2DE) or detected by a characteristic mass/charge ratio in a mass spectrometer (Figure 1). In the first proteomic investigation of Trx-target proteins in germinating barley embryo, Marx et al. (2005) used monobromobimane (mBBr) for visualization of proteins from barley embryo extracts reduced by Trx and separated by 2DE. Subsequently 16 target proteins including several α-amylase/trypsin inhibitors, chitinases, and cyclophilin, were detected in extracts of mature and germinating seeds using the more sensitive fluorescent cyanine dye Cy5 (Maeda et al., 2004). To detect specific disulfide targets in proteins separated by 2DE, a differential thiol-labeling procedure was developed and applied to proteins from mature barley seed extract (Maeda et al., 2005). Briefly, cysteines from disulfides reduced by Trx were blocked with IAM followed by full reduction by dithiothreitol (DTT) and alkylation by 4-vinylpyridine (4-VP). Following trypsin digestion, peptides containing cysteines reacted with IAM and 4-VP were distinguished by mass shifts of 57 and 105 Da, respectively (Figure 1B). Thus, nine disulfides mainly originating from α-amylase/protease inhibitors were identified as Trx substrates (Maeda et al., 2005).

A gel-free proteomics approach for Trx-target identification was developed based on isotope-coded affinity tags (ICAT) labeling followed by liquid chromatography–mass spectrometry (LC–MS) analysis (Hägglund et al., 2008). The ICAT reagents contain a thiol-reactive IAM group and isotope-coded linkers in “light” (ICATL) and “heavy” (ICATH) forms labeled with nine 12C and 13C carbon atoms, respectively. Since the only difference between ICATL and ICATH is the number of 12C/13C atoms, it is possible to quantify the labeling ratio in a mass spectrometer. Furthermore, the ICAT reagents contain a biontin tag for selective enrichment of labeled species. In order to adapt ICAT labeling for relative quantification of Trx-target disulfide reduction, samples were first incubated in the presence or absence of Trx followed by IAM quenching. Then remaining thiols were chemically reduced with tris(2-carboxyethyl)phosphine (TCEP) and labeled with ICATL and ICATH, respectively (Figure 2). The
FIGURE 1 | Procedures for identification of Trx h targets based on protein separation by two-dimensional gel electrophoresis. (A) Identification of target proteins using fluorescent dyes such as MBBR or Cy5. Fluorescence-scanned 2D-gels of mature barley seed proteins treated +/− Trx. The appearance of additional Cy5-labeled spots in the Trx-treated sample is indicated with circles. (B) Differential labeling procedure using iodoacetamide (IAM) and 4-vinylpyridine (4-VP) for identification of Trx h-reducible protein disulfides (top). Sections of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra for the tryptic peptide 141LMSCGDWCQDLGVFR155 (M+H = 1729.75 Da with reduced [−SH] cysteine residues) from α-amylase/subtilisin inhibitor BASI containing the Cys144-Cys148 disulfide exposed to differential thiol labeling (bottom). In control (the lower spectrum), a major peak at m/z 1939.9 is observed corresponding to the 4-VP pyridylethylated form of Cys144 and Cys148 (mass increase 2 × 105 Da). When treated with Trx (the upper spectrum), the base peak is shifted by 96 Da (2 × 57 Da − 2 × 57 Da) and appears at m/z 1843.8 instead corresponding to the IAM carbamidomethylated form of Cys144 and Cys148 (mass increase 2 × 57 Da), showing that these cysteines in BASI are involved in a Trx h-reducible disulfide. The minor peak at 1891.8 Da corresponds to a peptide containing one pyridylethylated and one carbamidomethylated cysteine residue, respectively (1 × 105 Da + 1 × 57 Da).
FIGURE 2 | Workflow for Trx h target identification using ICAT labeling and LC–MS/MS. Free reduced cysteine thiols (SH) are first blocked with IAM. Samples are incubated +/− Trx for reduction of target disulfides (S–S target) and treated with IAM to block free thiols released by Trx. Remaining oxidized thiols (S–S non-target) are then reduced using TCEP and free thiols are labeled with ICATL/ICATH. Following tryptic digestion, the ICAT-labeled peptides are enriched by avidin affinity chromatography and analyzed by LC–MS/MS for peptide identification and determination of ICATH/ICATL labeling ratios. ICATH/ICATL peptide ratios of 1 are expected for non-target disulfide bonds and ratios >1 are expected for peptides containing cysteines from disulfide bonds reduced by Trx. The ICAT approach was applied to extracts of dissected embryo and proteins released from aleurone layers resulting in the identification of more than 100 putative targets (Hägglund et al., 2008, 2010). The most extensively reduced target from barley embryo was dehydroascorbate reductase suggesting a possible link between the Trx system and the ascorbate/glutathione cycle.

MOLECULAR DETAILS OF TARGET RECOGNITION BY Trx h

The barley α-amylase/subtilisin inhibitor (BASI) contains two disulfide bonds located in the vicinity of the interfaces in contact with α-amylase and subtilisin, respectively. The disulfide close to the α-amylase surface was identified as a Trx h target by differential thiol labeling (Maeda et al., 2005) as outlined above (Figure 1B). To get further insight into the mechanism of Trx h-mediated reduction of this target disulfide, a complex of HvTrxh2 and BASI stabilized by an intermolecular disulfide bond was formed using single-cysteine mutants (HvTrxh2 C49S and BASI C144S). The structure of HvTrxh2-S–S-BASI at a resolution of 2.3 Å was determined by X-ray crystallography (Maeda et al., 2006). The complex is stabilized by numerous van der Waals contacts and three intermolecular hydrogen bonds involving the backbone of HvTrxh2 M88 and A106. This pattern of hydrogen bonds appears to be conserved among related thiol oxidoreductases in the Trx fold superfamily. To probe the importance of these hydrogen bonds, two HvTrxh2 variants M88P and A106P lacking the ability to form amide backbone hydrogen bond were constructed and assayed for activity toward target proteins and NTR. Enzyme kinetics indeed demonstrated that backbone hydrogen bonding involving A106 is important for interactions with BASI but appears not to affect reactivity with NTR (Björnberg et al., 2012). The M88P mutant was severely affected in terms of thiol reactivity and the role of M88 in target recognition could therefore not be conclusively demonstrated. Notably, an electrostatic contact between HvTrxh2 and BASI was engineered through a HvTrxh2 E86R mutation, which resulted in a threefold increase in disulfide reductase activity toward BASI (Björnberg et al., 2012).

Barley limit dextrinase inhibitor (LDI) contains nine cysteine residues forming four intramolecular disulfide bonds and a mixed disulfide with glutathione. Experiments with recombinant LDI in vitro revealed preferential reduction of the glutathionylated residue as well as complete disulfide reduction mediated by HvTrxh1 and HvTrxh2 (Jensen et al., 2012). Disulfide reduction correlates with loss of inhibitory activity proposed to occur due to conformational destabilization of reduced LDI.

PROSPECTS FOR INDUSTRIAL APPLICATIONS

Cereal crops are highly valuable for the nutrition of livestock due to the high percentage of carbohydrates, storage proteins, starch, fatty acids, and vitamins. Barley grains contain relatively low amounts of protein compared to other crops, for example legumes. Following germination, the protein reserves are mobilized by proteases released from the aleurone layer as well as by the pro-formed proteases already present in the endosperm. Overexpression of Trx in transgenic barley endosperm resulted in an increase in protein synthesis.
solubility (Wong et al., 2002). Trx was also used for modification of solubility of proteins in wheat endosperm (Wong et al., 2004). A plant-based “liquid feed” system showed that incubation with a functional NTR/Trx system increased the solubility of known Trx-target proteins (Sultan, A., Bjerg Christensen, J., Damgaard Poulzen, H., Svensson, B., and Finnie, C., 2009). The importance of this issue in regulating dormancy in barley. Plant Physiol. 150, 1006–1012.

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