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Functional Biotechnology

Secretory type of recombinant thioredoxin *h* induces ER stress in endosperm cells of transgenic rice

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ABSTRACT

Thioredoxin *h* (TRX *h*) functions as a reducing protein and is present in all organisms. As a new approach for inducing the endoplasmic reticulum (ER) stress, TRX *h* (OsTRX23) was expressed as a secretory protein using the endosperm-specific glutelin *GluB-1* promoter and a signal peptide. In transgenic rice seeds, the majority of the recombinant TRX *h* accumulated in the ER but some was also localized to the protein body IIs (PB-IIs). The rice grain quality was dependent on the TRX *h* accumulation level. Increased TRX *h* expression resulted in aberrant phenotypes, such as chalky and shriveled features, lower seed weight and lower seed protein content. Furthermore, the accumulation of some seed storage proteins (SSPs) was significantly suppressed and the morphology of the protein bodies (PB-Is and PB-IIs) changed according to the level of TRX *h*. SSPs, such as 13 kDa prolamin and GluA, were specifically modified via the reducing action of TRX *h*. These changes led to the activation of the ER stress response, which was accompanied by the expression of several chaperone proteins. Specifically, the ER stress markers BiP4 and BiP5 were significantly up-regulated by an increase in the level of TRX *h*. These results suggest that changes in the conformation of certain SSPs via the action of recombinant TRX *h* lead to an induced ER stress response in transgenic rice seeds.

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Introduction

Rice seed has been utilized as a bioreactor for recombinant protein or peptide production. Rice seed-based recombinant proteins are highly stable even when stored under room temperature conditions for several years. Additionally, the rice seed production system has advantages over the conventional expression systems using bacteria, insect and mammalian cells in terms of economy, scalability and safety (Takaiwa et al., 2008). Therefore, many kinds of transgenic rice that accumulate recombinant protein in the grains have been developed worldwide (Fujiwara et al., 2010; He et al., 2011), and transgenic rice plants that accumulate bioactive protein or peptides have been produced in our lab (Suzuki et al., 2012; Takagi et al., 2009; Wakasa et al., 2011a,b; Yasuda et al., 2006). However, the high accumulation of some recombinant proteins or peptides in rice seeds results in detrimental phenotypes, such as floury and shrunken features (Oono et al., 2010).

Recent data indicated that, in most cases, these detrimental phenotypes were due to the endoplasmic reticulum (ER) stress response (Oono et al., 2010; Wakasa et al., 2011c; Yasuda et al., 2009). The ER stress response is activated by the accumulation of unfolded or misfolded proteins in the ER. From studies of the ER stress response in mammalian and yeast cells, ER stress promotes the activation of at least three distinct intracellular signal transduction pathways. These pathways include (1) an abnormal protein refolding and degradation system, (2) inhibition of translation, and (3) apoptosis (Bertolotti et al., 2000; Harding et al., 1999; Okamura et al., 2000; Oyadomari and Mori, 2004; Yamaguchi et al., 2008).

An understanding of the molecular mechanisms that underlie the ER stress response is necessary if recombinant protein yields are to be increased in rice seeds. However, few reports concerning the ER stress response in rice seed have been published. In cultured cells and plant seedlings, ER stress is typically and easily induced via treatment with ER stress-inducible agents, such as dithiothreitol (DTT), which prevents disulfide bond formation, or tunicamycin, an inhibitor of Asn-linked glycosylation. Although treatments with these reagents can be easily administered to cultured cells and plant seedlings, these treatments are difficult to apply to developing rice seeds. Thus, new effective ER stress induction systems are crucial for the study of the ER stress response in developing rice seeds. Previously, ER stress mechanisms were analyzed using transgenic rice

Abbreviations: BPB, bromophenol blue; CBB, Coomassie brilliant blue; ER, endoplasmic reticulum; DTT, dithiothreitol; mBBr, monobromobimaine; PB, protein body; SSP, seed storage protein; TRX, thioredoxin.

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seeds that either overexpressed or suppressed the ER-chaperone protein BiP1 (Wakasa et al., 2011c; Yasuda et al., 2009). BiP1 is a key chaperone protein involved in ER quality control, and significant changes in BiP1 levels lead to ER stress induction in seeds. BiP1 overexpressing or BiP1 knock down transgenic rice may serve as good materials for studying the ER stress response in rice seeds.

The present study focused on thioredoxin (TRX) protein, since TRXs are involved in redox regulation in the cells of all organisms. The active form of TRX, the reduced TRX-$(SH)_2$ form, is involved in the disulfide bond reduction of substrate proteins. The oxidized form, Trx-S_2, is inactive and is reduced to TRX-$(SH)_2$ dithiol via NADPH and TRX reductases. TRX participates in many cellular processes such as carbon assimilation, seed germination, the self-incompatibility reaction, redox signaling, radical scavenging and detoxification (Gelhaye et al., 2004). In plants, TRX is usually encoded by a multigene family, and members of the TRX family are classified into 6 fundamental types (TRX *m*, TRX *f*, TRX *x*, TRX *y*, TRX *o* and TRX *h*) based on their conserved motifs, function and subcellular localization in cells. *Arabidopsis* contains at least 46 *TRX* (containing *TRX-like*) genes in its genome, which are classified into 17 subfamilies (Meyer et al., 2005). In the rice genome, thirty *TRX* genes have been identified (Nuruzzaman et al., 2008).

One TRX subgroup, TRX *h*, contains a small protein transit peptide. TRX *h*s share homology with other TRXs in the presence of the conserved catalytic site. The TRX *h* subgroup is further subdivided into three groups according to primary sequence and activity. Most TRX *h* proteins are constitutively expressed and localized to the cytosol (Gelhaye et al., 2004; Meyer et al., 2005), while a few TRX *h*s are detected in the phloem sap of rice (Ishiwatari et al., 1995; Sasaki et al., 1998). TRX *h* proteins are involved in plant development and stress responses, such as seed germination and early seedling development and oxidative stress (Gelhaye et al., 2004).

Interestingly, Buchanan's group generated transgenic barley plants that expressed wheat TRX *h* as a secretory protein in seeds. The wheat TRX *h* was expressed with the barley hordein signal peptide at the N terminus under the control of the endosperm-specific *hordein* promoter. Recombinant TRX *h* exhibited redox activity against seed proteins, resulting in increased soluble protein and accelerated germination after imbibition (Cho et al., 1999; Wong et al., 2002). Furthermore, similar results were also reported using transgenic wheat plants over-expressing or under-expressing TRX *h* by the same group (Li et al., 2009). Based on these results, recombinant TRX *h* reduced (inhibited) disulfide bond formation and disrupted the conformation of various proteins in barley seeds. Thus, in seed tissue, TRX *h* is expected to induce the ER stress response in a manner similar to DTT treatment. Nine of 30 isozymes of *TRX* were identified as TRX *h* (Nuruzzaman et al., 2008). In this study, an OsTRX *h* isozyme, OsTRX23, was expressed as a secretory protein at various levels using an endosperm-specific promoter in transgenic rice seeds and the relationship between TRX *h* accumulation levels and the ER stress response was examined. Transgenic rice seeds accumulating TRX *h* are expected to be useful for studies of the ER stress response in rice seed cells.

Materials and methods

Plant materials and production of transgenic rice

Rice (*Oryza sativa* L., cv. Kitaake) was used as the host for transformation. Thioredoxin *h* (TRX *h*) (*OsTRX23*) cDNA was inserted between the endosperm-specific *GluB-1* promoter with the signal peptide and *GluA-3* terminator of the expression cassette. The gene expression cassette was then cloned into the binary vector pGPTV-HPT (Becker, 1990) (Fig. 1). Plants were transformed via *Agrobacterium*-mediated transformation (Goto et al., 1999). Thirty

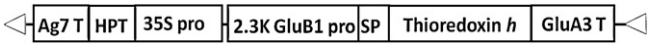

Fig. 1. Binary vector construct for the expression of TRX *h*. 2.3K GluB1 pro, 2.3 kb *glutelin B1* promoter; SP, glutelin B1 signal peptide; Thioredoxin *h*, *OsTRX23* coding region; GluA3T, *glutelin A3* terminator; 35S pro, *CaMV 35S* promoter; HPT, *hygromycin phosphotransferase* coding region; Ag7 T, *Agrobacterium gene 7* terminator.

independent transformants were generated and several transgenic plants with different TRX *h* accumulation levels were screened from the T1 populations.

Total protein extraction and immuno-blot analysis

Mature seeds from the transgenic rice line were harvested. Each grain was separately ground into a fine powder using a Multi-beads Shocker (YASUI KIKAI, Kyoto, Japan). For total protein extraction, 500 μL of extraction buffer [50 mM Tris–HCl (pH 6.8), 8 M urea, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue (BPB)] was added to the seed powder. The samples were vortexed for more than 1 h at room temperature. The mixture was centrifuged at 12,000 × *g* for 20 min at room temperature, and the crude soluble protein sample was decanted into a new tube. Total protein was subjected to immuno-blot analysis after electrophoresis on 12% SDS-PAGE. The approximate signal strength of each band was estimated using the National Institute of Health (NIH) ImageJ 1.43 program package.

Recombinant protein

The TRX *h* (OsTRX23) coding sequence was amplified by polymerase chain reaction (PCR). PCR products were inserted into pET23d (Novagen, Darmstadt, Germany). His-tagged recombinant proteins were expressed in *Escherichia coli* strain BL21-codon plus-RIL (Novagen). Subsequently, recombinant protein was purified on a Ni-NTA spin column (Qiagen, Chatsworth, CA). The purified sample was eluted using elution buffer [20 mM sodium phosphate buffer (pH 7.4), 500 mM NaCl, 500 mM imidazole]. The sample was exchanged the elution buffer with 100 mM potassium phosphate (pH 7.1) and 50% glycerol by ultrafiltration using Umplicon Ultra (Millipore, Billerica, MA). Samples were stored at −20 °C.

Extraction of TRX h from rice seed

Recombinant TRX *h* is present in the albumin/globulin fraction in rice seeds. Albumin/globulins were extracted from a premature seed using 500 μL of saline extraction buffer (0.5 M NaCl, 10 mM Tris–HCl pH 6.8). The mixture was centrifuged at 12,000 × *g* for 10 min at room temperature, and the crude soluble protein sample was decanted into a new tube. The crude protein sample was exchanged with saline extraction buffer containing 100 mM potassium phosphate (pH 7.1) and 50% glycerol by ultrafiltration using Amicon Ultra (Millipore). Samples were stored at −20 °C.

Seed protein content

Total seed proteins were extracted by using a modified total protein extraction buffer [50 mM Tris–HCl (pH 6.8), 4 M urea, 4% SDS, 5% 2-mercaptoethanol]. Seed protein content was measured by the RC DC protein assay kit (BIO-RAD) according to the manufacturer's instructions.

Measurement of TRX h activity

TRX *h* activity was measured using the insulin-reduction assay (Cazalis et al., 2006). Briefly, 1 mL of reaction mixture (100 mM

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