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Method for enhancement of plant redox-related protein expression and its application for *in vitro* reduction of chloroplastic thioredoxins

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ABSTRACT

Plant redox-related proteins were overexpressed using a genetic codon substitution downstream of the translation initiation codon. This method significantly improved recombinant protein expression levels of *Arabidopsis* chloroplastic thioredoxins and cytosolic nicotinamide adenine dinucleotide phosphate (NADPH)-dependent thioredoxin reductase (E.C. 1.8.1.9) in *Escherichia coli*. Using these proteins, the *in vitro* chloroplastic thioredoxins-reduction system was reconstituted in an NADPH-dependent manner. This system could convert the five classes of chloroplastic *Arabidopsis* thioredoxins and two chloroplastic Spinach thioredoxins to their reduced forms, independent of dithiothreitol and the photosynthetic electron transport system.

Introduction

Thioredoxin (Trx) is a small ubiquitous protein that mediates the disulfide–dithiol exchange reaction [1–3]. In the reduced state, Trxs are able to reduce disulfide bonds of target proteins, thereby modulating their activity. In plants, the Trx-dependent redox regulatory system operates in various organelles including the cytoplasm, mitochondria, and chloroplasts [4–7]. Moreover, a number of Trx-target proteins have been identified in various organelles [8–14], and it has been shown that these thioredoxins play a key role in plant redox regulation.

Plant Trxs are reduced by different mechanisms in each organelle. Cytoplasmic and mitochondrial Trxs receive reducing equivalents via nicotinamide adenine dinucleotide phosphate (NADPH) and NADPH-dependent thioredoxin reductase (NTR) [15]. In contrast, chloroplastic Trxs are reduced through ferredoxin (Fd) and ferredoxin–thioredoxin reductase (E.C. 1.8.7.2) with electrons provided by photosynthetic electron transport in a light-dependent manner [16–18].

Plant redox-related proteins contain cofactors, such as a flavin adenine dinucleotide (FAD) molecule in *Arabidopsis* NTR [19]. It is well established that eukaryotic proteins having cofactors are difficult to overexpress in bacteria [20]. Here, we report that introducing A/T-rich sequences downstream of the translation initiation codon leads to overexpression of recombinant plant redox-related proteins in *Escherichia coli*. Using this system allowed us to reconstitute a simple *in vitro* reduction system of chloroplastic Trxs without the photosynthetic electron transport chain, by purified recombinant plant redox-related proteins.

Materials and methods

Cloning, expression, and purification of Arabidopsis NADPH-dependent thioredoxin reductase

The mature form of *Arabidopsis* NTRA protein was predicted by the TargetP 1.1 program [21]. The NTRA gene (At2g17420) was obtained by polymerase chain reaction (PCR) amplification from an *Arabidopsis* cDNA library [11], using the following oligonucleotides: 5′-aactgcagcatatggccgccgccgccgtcgacat-3′ (*Nde*I) and 5′-cggaattcaatcactcttaccctcct-3′ (*Eco*RI). Restriction sites for the enzyme shown in parentheses are underlined. The amplified DNA fragments were cloned into the *Nde*I and *Eco*RI sites of pET23a (Merck Millipore, Darmstadt, Germany) and the DNA sequences were confirmed. The NTRA (codon substitution) gene was amplified by PCR amplification from *Arabidopsis* NTRA/pET23a plasmid, using the following oligonucleotides: 5′-aactcgagcatatggcag ctgcagctgttgatatggaaactcacaaaaccaaggtt-3′ (*Nde*I) and 5′-cggaattc aatcactcttaccctcct-3′ (*Eco*RI). Amplified DNA fragments were then cloned into the *Nde*I and *Eco*RI sites of pET23a and the DNA sequences were confirmed. The recombinant NTRA (codon substitution) was expressed in *E. coli* BL21 (DE3) cells and purified as

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follows: *E. coli* cells were suspended in 25 mM Tris–HCl (pH 7.5), and disrupted by sonication (SONIFIER 250, BRANSON, Danbury, Connecticut) at 4 °C. Disrupted cells were centrifuged at 100,000×*g* for 40 min and the supernatant (crude extract) was applied to a DEAE-TOYOPEARL® 650 M column (Tosoh, Tokyo, Japan), previously equilibrated with 25 mM Tris–HCl (pH 7.5). Proteins were then eluted with a 0–150 mM linear gradient of NaCl in 25 mM Tris–HCl (pH 7.5). The peak fractions containing NTRA were collected and solid ammonium sulfate was added to obtain a final concentration of 1.3 M. The solution was then applied to a Butyl-TOYOPEARL® 650 M column (Tosoh, Tokyo, Japan), and eluted with a 1.3–0 M inverse gradient of ammonium sulfate in 25 mM Tris–HCl (pH 7.5). The yellowish NTRA peak fractions were collected, and dialyzed against 25 mM PIPES-NaOH (pH 6.3) and 5 mM $MgCl_2$. The dialyzed protein solution was applied to AF-Blue-TOYOPEARL® 650ML (Tosoh, Tokyo, Japan), and eluted with a 0–300 mM linear gradient of NaCl in 25 mM PIPES-NaOH (pH 6.3), 5 mM $MgCl_2$. The peak yellowish NTRA fractions were collected and stored at −80 °C.

Cloning, expression, and purification of Arabidopsis Trx-f1 and -f2

The mature form of *Arabidopsis* Trx-*f*1 (At3g02730) and -*f*2 (At5g16400) proteins have been previously predicted [22]. Trx-*f*1 and Trx-*f*2 genes were obtained by PCR amplification from an *Arabidopsis* cDNA library [11], using the following oligonucleotides: 5′-aactgcagcatatggttggtcaggtgacggaggtcgat-3′ (*Nde*I, Trx-*f*1), 5′-cgcggatcctcatccggaagcagcagacct-3′ (*Bam*HI, Trx-*f*1), 5′-aactgcag catatggttggccaggtgacggaggttgat-3′ (*Nde*I, Trx-*f*2) and 5′-cggaattca gcctgaccttgctgctt-3′ (*Eco*RI, Trx-*f*2). Restriction sites for the enzymes shown in parentheses are underlined. The amplified DNA fragments were cloned into the *Nde*I and *Bam*HI (or *Eco*RI) sites of pET23a and DNA sequences were confirmed. The Trx-*f*1 and -*f*2 (codon substitution) genes were amplified by PCR from *Arabidopsis* Trx-*f*1/pET23a and Trx-*f*2/pET23a plasmids, using the following oligonucleotides: 5′-aactgcagcatatggttggtcaagttactg aagttgataaagacacgttctggcccatcgtt-3′ (*Nde*I, Trx-*f*1), 5′-cgcggatcctc atccggaagcagcagacct-3′ (*Bam*HI, Trx-*f*1), 5′-aactcgagcatatggttggt caagttactgaagttgataaagacacgttctggccaattgtt-3′ (*Nde*I, Trx-*f*2), and 5′-cggaattcaatcactcttaccctcct-3′ (*Eco*RI, Trx-*f*2). Amplified DNA fragments were then cloned into the *Nde*I and *Bam*HI (or *Eco*RI) sites of pET23a and DNA sequences were confirmed. The recombinant Trx-*f*1 and -*f*2 (codon substitution) were expressed in *E. coli* BL21 (DE3) cells and purified as described previously [23], except that dithiothreitol (DTT) was removed. The peak protein fractions were collected and stored at −80 °C.

Protein preparation

Arabidopsis Trx-*m*1, -*m*2, -*m*4, -*x*, -*y*1, -*y*2, and -*z* without any affinity tags were overexpressed in *E. coli* [24], and purified by conventional column chromatography, as described previously [23]. *Arabidopsis* Trx-*m*3 was overexpressed with *Strep*-tag®II at the C-terminus, and purified by *Strep*-Tactin Sepharose (IBA GmbH, Goettingen, Germany) [25,26]. Spinach Trx-*f* [27] and Trx-*m* [23,28] were prepared as previously described.

Insulin reduction assay

To check the disulfide reduction activity of recombinant Trx-*f*1 and Trx-*f*2, the change in turbidity of the insulin solution due to precipitation of free insulin B chain by reduction was measured spectrophotometrically at 650 nm [27,29]. The assay mixture contained 100 mM potassium phosphate (pH 7.0), 2 mM EDTA, and 130 μM bovine insulin. The reaction was initiated by adding 330 μM DTT into the assay mixture, and the change in turbidity was monitored at 650 nm at 25 °C.

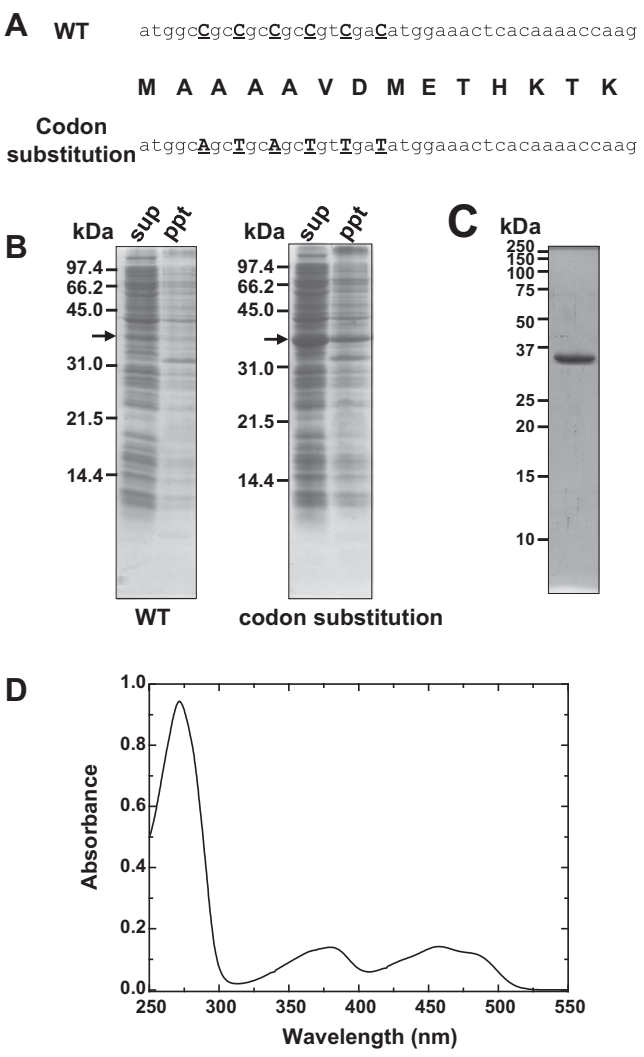

Fig. 1. Overexpression and purification of *Arabidopsis* NADPH-dependent thioredoxin reductase (NTRA). (A) Codon substitution to A/T-rich sequences downstream of the translation initiation codon of the *Arabidopsis* NTRA-encoded gene. Substituted nucleotides are indicated with underlined capital letters. (B) Overexpression of the NTRA protein in *E. coli*. Left panel, lysate from *E. coli* carrying the wild-type (WT) gene; Right panel, lysate from *E. coli* carrying the codon substitute gene. Sonicated cell lysates were centrifuged, and supernatants (sup) and precipitates (ppt) of the lysate were subjected to SDS–PAGE. (C) Purified *Arabidopsis* NTRA protein. The NTRA protein was purified by conventional column chromatography as described in the "Materials and methods" section, and subjected to SDS–PAGE. (D) Absorption spectrum of the *Arabidopsis* NTRA (6 μM, as dimer) in 25 mM Tris–HCl (pH 7.5). The absorption coefficient value of FAD was calculated as ε_{450} = 11,300 M^{-1} cm^{-1}.

Light-independent chloroplast thioredoxin reduction assay

Chloroplastic thioredoxins (9 μM) were incubated with cytosolic NTRA (50 nM) and NADPH (1 mM) in 25 mM Tris–HCl (pH 7.5), for 60 min at 25 °C. To determine redox states of disulfide bonds in thioredoxins, free sulfhydryl groups were modified with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS)[2]

[2] *Abbreviations used:* AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; DTT, dithiothreitol; FAD, flavin adenine dinucleotide; Fd, ferredoxin; NADPH, nicotinamide adenine dinucleotide phosphate; NTR, NADPH-dependent thioredoxin reductase; PCR, polymerase chain reaction; Trx, thioredoxin; WT, wild-type.

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