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# Orange protein, phytoene synthase regulator, has protein disulfide reductase activity

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**ABSTRACT**

Orange protein (OR) is known to interact with phytoene synthase (PSY) that commits the first step in carotenoid biosynthesis, and functions as a major post-transcriptional regulator on PSY. We here tried to reveal enzymatic characteristics of OR, that is, protein disulfide reductase (PDR) activity of the *Arabidopsis thaliana* OR protein (AtOR) was analyzed using dieosin glutathione disulfide (Di-E-GSSG) as a substrate. The AtOR part containing only the zinc (Zn)-finger motif was found to show PDR activity, with an apparent $K_m$ of 12,632 nM, $K_{cat}$ of 11.85 $min^{-1}$, and $K_{cat}K_m^{-1}$ of $15.6 \times 10^3$ $M^{-1}sec^{-1}$. To evaluate the significance of the N-terminal region of AtOR, we examined the kinetic parameters of a fusion protein composed of the N-terminal region and the Zn-finger motif from AtOR. Consequently, the fusion protein had lower values for $K_m$ (2,074 nM) and $K_{cat}$ (3.18 $min^{-1}$) and higher catalytic efficiency ($25.9 \times 10^3$ $M^{-1}sec^{-1}$) than that of only the Zn-finger motif part, suggesting that the N-terminal region of AtOR should be important for substrate affinity and catalytic efficiency of PDR activity. Complementation experiments with *E. coli* further demonstrated that AtOR containing the N-terminal region and the Zn-finger motif increases phytoene synthase activity of AtPSY especially under reduced circumstances retaining a NADPH- and $H^+$-regeneration system.

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## Introduction

Carotenoids are diverse color pigments naturally occurring in plants and algae, as well as parts of fungi, bacteria, and archaea. They play important roles in development, photosynthesis, root-mycorrhizal interactions, and the production of phytohormones, such as abscisic acid and strigolactones.[1] Phytoene synthase (PSY) catalyzes the formation of phytoene (15-*cis*-phytoene) from geranylgeranyl diphosphate (GGPP) as the first committed step in carotenoid biosynthesis and is one of the most important regulators in the carotenoid biosynthesis.[2,3] The *Orange* (*OR*) gene was isolated from an orange cauliflower mutant (*Brassica olerancea* var. *botrytis*) that accumulates ß-carotene in organs that normally do not contain carotenoids.[4] It was found that PSY and the OR protein (OR) physically interacted with each other in plastids, and OR served as the major post-transcriptional regulators on PSY.[2] It was furthermore shown that the N-terminal region of the BoOR protein interacted with PSY.[2] The *Arabidopsis thaliana OR* gene (*At5g61670*) encodes a protein (AtOR) of 307 amino acids with a putative molecular weight of 33,789. It contains 11 cysteine residues and has two predicted zinc (Zn) finger-like motifs (CXXCXGXG), Zn1 and Zn2 (Supplementary data). The two Zn finger motifs of AtOR are similar to the C4-type Zn finger motifs of the *E. coli* DnaJ and AtBSD2 proteins.[5–8] In chloroplasts, four other proteins likewise contain the Zn-finger motifs: CYO1/SCO2,[9–11] LQY1[12] and HCF222[13] in/at the thylakoid membrane, and PSA2 in the thylakoid lumen.[14] They all exhibit protein-disulfide reductase (PDR) activity like DnaJ.[15] We thus tested whether AtOR retains PDR activity with a protein disulfide reductase (PDR) assay.[8,10,16] In this assay, fluorescence increases if the reduction of disulfide bonds is mediated by dieosin glutathione (Di-E-GSSG). The present study reveals the enzymatic characteristics of AtOR as PDR.

## Materials and methods

### *Expression and purification of truncated AtOR proteins*

An expression vector (pET-#0219) that contains the cDNA fragment encoding amino acids between 45 and 307 of the wild-type AtOR (At5g61670) protein from *Arabidopsis thaliana* was amplified by RT-PCR using primers, P217 and P227 (Figure 1). The amplified DNA fragments were ligated with the expression vector pET24a (+) digested with *Nde*I and *Xho*I using In-Fusion HD cloning kit (Takara bio, Japan). cDNA fragments encoding amino acids between 45 and 95, and between 220 and 307 of AtOR were amplified by RT-PCR using primer sets of P227 and P229, and of P228 and P217, respectively. These DNA fragments were ligated with the expression vector pET24a(+) digested with *Nde*I and *Xho*I using In-Fusion HD cloning kit, designated pET-#0220. The amplified DNA fragments using primers P206 and P217 were ligated with pET24a (+), designated pET-#0213. The sequences of the DNA fragments were confirmed. The *E. coli* BL21-CodonPlus (DE3; Agilent Technologies, U.S.

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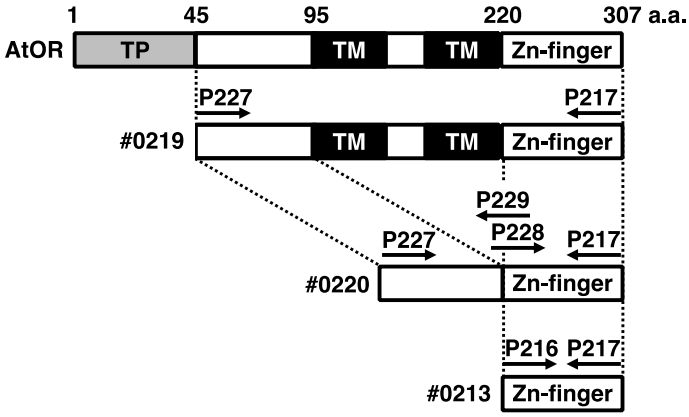


Figure 1. Schematic presentation of AtOR (At5g61670) protein and variants. The positions of AtOR truncations are indicated. TP, transit peptide; TM: transmembrane domain; Zn-finger: zinc finger motif. Arrows show the position of the primers used in the PCR reactions.

A.) was used to express the OR fusion proteins with 6× His tags. The expression and purification of these proteins were performed as described.[9]

### Preparation of Di-E-GSSG and assay for PDI-dependent disulfide reduction

Di-E-GSSG was prepared as described.[16] PDI disulfide reduction activity was monitored in PDI assay buffer (100 mM potassium phosphate, pH 7.0) containing OR protein (100 nM) and Di-E-GSSG (188–4700 nM) with or without DTT (5 μM) based on the increased fluorescence at 545 nm with excitation at 525 nm.[16] The kinetic parameters were calculated using KaleidaGraph software (Synergy software, U.S.A.).

### Bacterial strains and plasmid construction

The plasmid pAC-HIEI was constructed for the expression of the *Pantoea ananatis crtE* and *crtI* genes in addition to the *Haematococcus pluvialis IDI* gene by eliminating *crtB* encoding phytoene synthase from the plasmid pAC-HIEBI that produces lycopene in *E. coli*.[17] The plasmids pCDF-AtPSY, pCDF-gdh-AtPSY and pCDF-zwf-AtPSY were constructed by inserting the *AtPSY* gene fragment into pCDF, pCDF-gdh, and pCDF-zwf, respectively.[18] The #0213, #0219, #0220 fragments were inserted into pET24a creating pET-#0213, pET-#0219, pET-#0220, respectively.

As *E. coli* strain as the host, we used BL21(DE3). The transformed *E. coli* cells were cultured in 2YT medium (1.6% Bactotryptone, 1.0% yeast extract, 0.5% NaCl) containing antibiotics, ampicillin (75 mg $L^{-1}$), tetracycline (12.5 mg $L^{-1}$) and spectinomycin (100 mg $L^{-1}$), at 37°C. Then, we inoculated this preculture into the new 2YT medium with the antibiotics and 0.05 mM IPTG, and cultured at 20°C for 2 days.

### Analysis of carotenoids accumulated in recombinant E. coli

We extracted and analyzed the carotenoids as described.[19]

## Results and discussion

To elucidate the functional differences in each region of AtOR, we generated three expression vectors encoding truncated AtOR proteins. #0219 is the expression vector encoding amino acid 45–307 of the full length of AtOR except for the putative transit peptide, the putative mature AtOR protein (Supplemental data). #0220 and #0213 are the expression vectors from #0219 without the putative transmembrane region, "95–220 amino acids" and encoding only Zn-finger motif of 220–307 amino acids, respectively. We tried to synthesize the putative mature AtOR protein (#0219) in *E. coli*, but it was not observed under all *E. coli* growth/IPTG concentration conditions we tried (Supplemental data). Therefore, we expressed the truncated *AtOR* genes that coding for the putative mature protein deleting the transmembrane region (#0219) and only the Zn-finger motif (#0213). These two truncated proteins were soluble and could be used for protein disulfide reductase assay using Di-E-GSSG at a substrate.[8,10] In the sample incubated with Di-E-GSSG and dithiothreitol (DTT), the close proximity of the eosin moieties resulted in self-quenching,[16] and the molecule had relatively low fluorescence at an excitation of 525 nm.[10] In the sample incubated with Di-E-GSSG, DTT and the AtOR protein (#0213), the two eosin moieties were spatially separated because of the reduction of disulfide bonds of Di-E-GSSG, and fluorescence increased.[10] Time course analyses of the reduction of Di-E-GSSG with AtOR protein (#0213) and DTT, but not with DTT alone (Figure 2(a)), suggesting that AtOR protein has PDR activity. To estimate $K_m$ and $K_{cat}$ of AtOR proteins, we incubated AtOR proteins with DTT and 500–7,000 nM Di-E-GSSG at 25°C and monitored initial rates of eosin glutathione (E-GSSG) formation (Figure 2(b)). The reductase activity of AtOR (#0213) increased as a function of Di-E-GSSG, with a $K_m$ of 12,632 ± 6,178 nM and $V_{max}$ of 1185 ± 407 nM $min^{-1}$. Therefore, we estimated the $K_{cat}$ and catalytic efficiency ($K_{cat}K_m^{-1}$) as11.85 ± 4.07 $min^{-1}$ and 15.6 ± 1.2 × $10^3$ $M^{-1}sec^{-1}$, respectively (Figure 3). To analyze the significance of the N-terminal region of AtOR, we estimated the kinetic parameters of AtOR (#0220)(Figure 2(b)). The $K_m$, $K_{cat}$ and $K_{cat}K_m^{-1}$ of AtOR (#0220) were 2,074 ± 246 nM, 3.18 ± 0.14 $min^{-1}$, and 25.9 ± 3.3 × $10^3$ $M^{-1}sec^{-1}$, respectively (Figure 3). The $K_m$ of AtOR (#0220) was about 6-fold lower than that of AtOR (#0213), suggesting the N-terminal region of AtOR should be important for substrate affinity. The catalytic efficiency of AtOR (#0220) was higher than that of AtOR (#0213). The results indicated that the N-terminal region of AtOR should promote the catalytic efficiency of PDR activity.

The holdase activity of sweetpotato (*Ipomoea batatas*) OR protein (IbOR) protected the stability of IbPSY protein and enhanced heat and oxidative stress tolerance in plants.[3] IbOR protein inhibited oxidative stress-induced aggregation of IbPSY *in vitro*. The treatment of IbPSY with $H_2O_2$ induced aggregation but the presence of IbOR protein inhibits the aggregation of IbPSY protein.[3] Since AtPSY protein has six cysteine residues, PSY protein should form disulfide bound

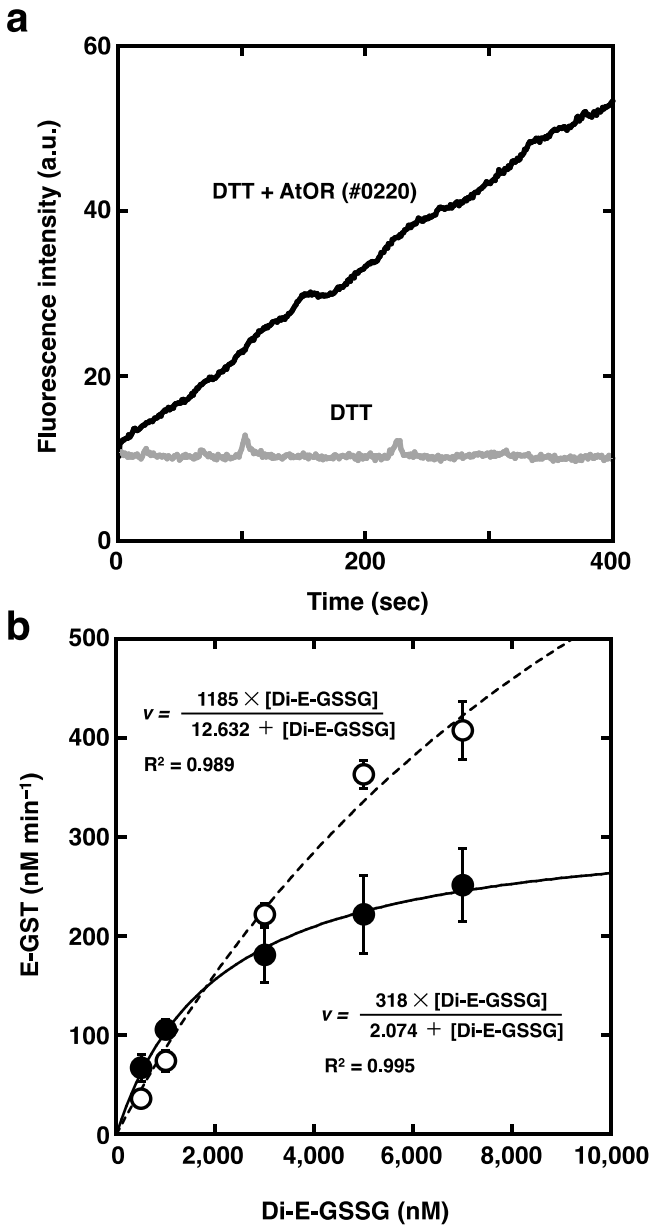


**Figure 2.** PDR activity of AtOR protein. (a) Di-E-GSSG was incubated with DTT at 25°C in the presence (black line) or absence (gray line) of AtOR (#0213). The fluorescence was recorded with excitation at 525 nm and emission at 545 nm. (b) Michaelis-Menten curves for PDR kinetics with 5 μM DTT of AtOR (#0213)(open circles and broken line) and AtOR (#0220)(closed circles and solid line). AtOR and DTT were incubated with varying concentrations of Di-E-GSSG at 25°C, and initial rates of E-GSH formation were monitored as a function of the concentration of Di-E-GSSG. Values represent the mean ± S.D. ($n$= 3).

| | $K_m$ (nM) | $K_{cat}$ ($min^{-1}$) | $K_{cat}\ K_m^{-1}$ ($\times 10^3\ M^{-1}sec^{-1}$) |
|---|---|---|---|
| AtOR (#0220) | 2,074 ± 264 | 3.18 ± 0.14 | 25.9 ± 3.3 |
| AtOR (#0213) | 12,632 ± 6,178 | 11.85 ± 4.07 | 15.6 ± 1.2 |
| AtBSD2 | 9,189 ± 829 | 3.39 ± 0.16 | 6.2 ± 0.8 |

**Figure 3.** Kinetic properties of AtOR and AtBSD2. Data for AtBSD2 was obtained in the previous study.[8]

under oxidative stress and the enzymatic activity is reduced, however the PDR activity of OR protein suppresses the oxidative inactivation of PSY. This hypothesis requires further investigation.

We further performed complementation experiments with *E. coli* to examine the effect of AtOR toward phytoene synthase activity of the AtPSY protein (Figure 4). When AtOR coexisted with AtPSY in the pAC-HIEI-carrying *E. coli*, which further

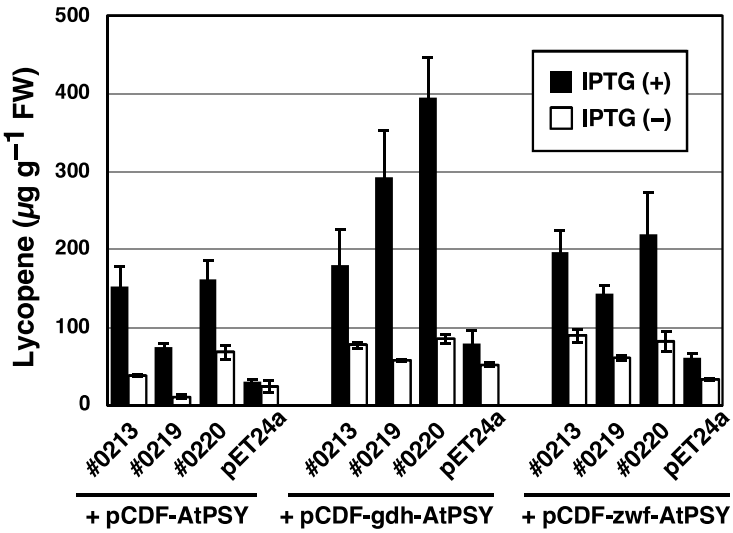


**Figure 4.** Lycopene production by *E. coli* BL21(DE3) that carried pET-#0213, pET-#0219, pET-#0220 or pET24a, and pCDF-AtPSY, pCDF-gdh-AtPSY or pCDF-wzf-AtPSY, in addition to pAC-HIEI. Values represent the mean ± S.D. ($n$ = 3).

includes pET-#0213, pET#0219 or pET-#0220 and pCDF-AtPSY, lycopene content was increased several times. As for the *E. coli* transformants possessing pET#0219 and pET-#0220, lycopene amounts were further elevated in the presence of the *Bacillus subtilis* glucose dehydrogenase (*gdh*) gene to supply sufficient levels of NADPH and $H^+$. This result indicated that AtOR containing the N-terminal region and the Zn-finger motif increases phytoene synthase activity of AtPSY especially under reduced circumstances having a NADPH- and $H^+$-regeneration system. We also reported that when the *gdh* and *zwf* genes, as the NADPH-regenerating enzyme genes, were introduced into the ß-amyrin-producing *E. coli*, the productivity was increased only in the case of *gdh*.[18]

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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