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Post-translational modifications to catalytic cysteine by selenium enhance the enzyme activity of glyceraldehyde-3-phosphate dehydrogenase from *Brassica oleracea var. italica*.

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Abstract

Selenium (Se) was recently shown to be beneficial for plants, and its application to crop production, drug development, and environmental hygiene is expected. The enzyme activities of some proteins were previously reported to be enhanced in plants grown with Se. These increases may be attributed to the binding of Se to catalytic cysteine by post-translational modifications (PTMs), which is a novel mechanism of expression. Therefore, the present study investigated Se binding and increases in enzyme activity by PTMs to cytoplasmic glyceraldehyde-3-phosphate dehydrogenase (GAPC) from broccoli (*B. oleracea var. italica*), which is classified as a Se-accumulating species, *in planta* and *in vitro*. GAPC derived from plants cultivated with 1 μM selenate had more Se bonds and stronger enzyme activity than that from those cultivated without selenate. BES-Thio, a fluorescent probe that identifies thiol or selenol groups, revealed that increases in GAPC activity by Se binding were due to the formation of a selenol group, which is more reactive than a thiol group, on GAPC-catalyzed cysteine by PTMs. Furthermore, purified recombinant GAPC and mutants (C156S, C160S, and C156S/C160S) were reduced and reacted with GSSeSG *in vitro* to investigate Se binding and selenol group generation by PTMs to recombinant GAPC and mutant proteins. The results obtained show that Se binding and selenol group generation by PTMs occurred only at Cys 156, which corresponds to the catalytic Cys. In addition, V_{max}, K_{cat}, and K_{cat}/K_m values of Se binding GAPC synthesized *in vitro* using purified BoGAPC were 1.49-, 1.48-, and 1.86-fold higher, respectively, than those of GAPC. These results indicate that the generation of selenol group at the catalytic Cys of GAPC by PTM improves the enzyme activity.

Key words:

Selenium, Post-translational modification, Glyceraldehyde-3-phosphate dehydrogenase, *Brassica oleracea var. italica*, Selenylation

Statements about Conflict of Interest:

The authors declare no conflict of interest associated with this manuscript.

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Introduction

Selenium (Se) is an essential trace element for most organisms, except higher plants, and is a key component of selenoproteins [1]. Since selenoproteins are involved in

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a number of biological functions, mainly as antioxidants, Se deficiency causes lethal diseases through selenoprotein dysfunction [2]. However, Se species exhibit high reactivity *in vivo*, with excessive accumulation being harmful to organisms [3]. Higher plants cannot synthesize selenoproteins and do not require Se to sustain life [4]. Se is recognized as a toxic element because its accumulation is cytotoxic.

Recent research on the application of Se to crop production, phytoremediation, and medicine development revealed its dynamics in higher plants [5]. A low Se concentration exerted diverse beneficial effects on plant growth [6]. Furthermore, various species of plants cultivated with Se exhibited stronger resistance to environmental stresses, such as drought, low temperatures, excessive light, heavy metal toxicity, and pathogens [6-8]. The beneficial effects of Se have been attributed to its regulation of gene expression, and the main target is the antioxidant system [9]. However, many details, including the signaling pathway of Se and the mechanisms underlying beneficial effects due to other factors, currently remain unclear.

We previously revealed that cultivation with trace Se enhanced or modified the functions of specific proteins. In *Chlamydomonas reinhardtii*, Se supplementation induced H_2O_2 reduction activity in glutathione peroxidase homolog (GPXH) [10]. GPXH is an isozyme of glutathione peroxidase (GPX), which is a selenoprotein, which has Cys instead of Sec at its active site, and originally does not exhibit H_2O_2 reduction activity. Verification using several types of mutant GPXH demonstrated that the activation of GPXH was caused by Se binding through post-translational modifications (PTMs) to Cys-38 at the GPXH active site [11]. In *Arabidopsis thaliana*, trace selenate (SeO_4^{2-}) enhanced the enzyme activity of cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPC), a glycolytic enzyme with catalytic Cys at its active site, and positively affected plant growth [12]. These findings suggest that Se-induced increases in GAPC activity were due to stronger nucleophilic reactivity as a result of the formation of a selenol group (-SeH) by a Se bond to the thiol group (-SH) of catalytic Cys through PTMs. This phenomenon is also predicted to be involved in the mechanisms underlying the beneficial effects of trace amounts of Se on plants, which have not yet been examined in detail. However, the mechanisms by which Se binds to a protein and enhances enzyme activity in plants that do not incorporate Se as Sec during protein translation remain unclear.

We herein propose that Se-induced increases in enzymatic activity are attributed to the conversion of the catalytic Cys thiol group to a selenol group by Se binding via PTMs. In support of this hypothesis, we refer to the nature of Se in enzymatic activity and Se binding by PTMs to non-Se proteins. Sec is an important feature of selenoproteins and is the 21st amino acid at which sulfur in Cys is replaced by Se [13]. The selenol group side chain ($pK_a = 5.2$) of Sec exhibits stronger nucleophilic reactivity under physiological conditions than the thiol group ($pK_a = 8.5$) [14]. Formate dehydrogenase (FDH), a wild-type selenoprotein that contains catalytic Sec, had a higher catalytic constant (K_{cat}) than mutant FDH, in which Sec replaced Cys [15]. The formation of Se-linked catalytic Cys by PTMs has been demonstrated in experiments using bovine liver-derived rhodanese, a protein with catalytic Cys at its active site. *In vitro* analyses revealed that a reaction with glutathione selenotrisulfide (GSSeSG) resulting from the reduction of selenite by glutathione (GSH) was responsible for the conversion of the thiol group in catalytic Cys to a selenol group [16]. Furthermore, reversible PTMs of the Cys residue, such as oxidation, S-nitrosylation, and S-hypersulfhydration, have been shown to function as intracellular signals and modify protein functions. S-sulfhydration, the conversion of the thiol group (Cys-SH) of catalytic Cys to a persulfide group (Cys-SSH), is expected to be a similar phenomenon to that of PTMs by Se due to similarities in the chemical properties of S and Se [17, 18].

The present study investigated Se binding to proteins by PTMs, with a focus on the mechanisms underlying Se binding to catalytic Cys and the resulting enhancements in enzyme activity. *In planta* and *in vitro* experiments were performed using GAPC purified from *Brassica oleracea var. italica*, which has a higher Se accumulation capacity than *Arabidopsis* [19], and its recombinant and mutant forms.

Materials and methods

Plant materials and growth conditions

Broccoli seeds (*B. oleracea var. italica*) were purchased from Nakahara Seeds Product Co., Ltd. (Hakata, Fukuoka, Japan). They were sterilized with NaClO (1% active chlorine) for 10 min, washed twice with sterile water, and

germinated on absorbent cotton medium impregnated with 4.3 g/L Murashige and Skoog basal salt mixture with or without 1 μM selenate in a plant box. Seeds were initially subjected to low temperature conditions (4°C in the dark) for 72 h, followed by dark conditions (room temperature in the dark) for 72 h. Seeds germinated under these conditions were grown in a growth chamber (23°C, 14/10-h light/dark cycle) for 3 days. These steps were performed aseptically, and sterilization was achieved by autoclaving at 121°C for 15 min.

Plasmid construction for the expression of BoGAPC and BoGAPC mutants

Synthetic genes encoding BoGAPC were obtained from Integrated DNA Technologies (IDT) (Coralville, IA, USA). Synthetic genes encoding the single (BoGAPC-C156S and -C160S) and double (BoGAPC-C156S/C160S) mutants of BoGAPC were also obtained from IDT. The EnsemblPlants (http://plants.ensembl.org/index.html) Gene ID for BoGAPC is Bo5g017500. The nucleotide sequences of the genes were optimized for higher expression in *Escherichia coli* without changing the amino acid sequences. The expression vectors for BoGAPC and BoGAPC mutants, pRham-BoGAPC and pRham-BoGAPC mutants (pRham-BoGAPC-C156S, pRham-BoGAPC-C160S, and pRham-BoGAPC-C156S/C160S), were constructed using the Expresso Rhamnose Cloning and Expression System, N-His in accordance with the manufacturer's instructions. The expression of BoGAPC and BoGAPC mutants in *E. coli* 10 G was performed as follows. *E. coli* cells harboring pRham-BoGAPC or pRham-BoGAPC mutants were grown in LB_broth with 50 μg/mL kanamycin to OD600 =0.8 before induction with 0.2 % (w/v) rhamnose. Growth was then continued at 18 °C for 24 h.

Measurement of GAPC activity

Three hundred milligrams of plant material was homogenized in 1 mL of 50 mM potassium phosphate buffer (pH 8.0) containing 1 mM EDTA, 2.5 mM dithiothreitol (DTT), 1 mM GSH, 1% (w/v) polyvinylpyrrolidone, and 10% (v/v) glycerol using a mortar and pestle. The homogenate was centrifuged at 12,000 × *g* for 10 min and the supernatant was collected as a crude enzyme solution. NAD-dependent GAPC (EC 1.2.1.12) was measured as the increase in absorbance at 340 nm due to the oxidative reduction of NAD^+ to NADH. The reaction mixture contained the enzyme preparation, 0.3 mM glyceraldehyde-3-phosphate (G3P), and 0.15 mM of NAD^+ in 120 μL of assay buffer (15 mM pyrophosphate buffer, 30 mM sodium arsenate, and 2 mM DTT at pH 8.5). The increase in absorbance at 340 nm at 25°C was monitored for three minutes using a microplate reader. All experiments and assays were performed in triplicate.

Purification of GAPC and recombinant proteins

The purification procedure for GAPC was as follows: the crude enzyme solution was centrifuged at 12,000 × *g* for 30 min and the supernatant was collected. Ammonium sulfate was then dissolved in the solution to 30% saturation. After being left to stand for 30 min, the resulting precipitate was removed by centrifugation and the supernatant was collected for the next step. The enzyme solution was charged onto a HiTrap Phenyl FF high sub column (GE Healthcare) previously equilibrated with buffer A (50 mM potassium phosphate, pH 7.5, 10% (w/w) glycerol, 2.5 mM DTT, 1 mM GSH, and 1 mM EDTA) containing 30% saturated ammonium sulfate, followed by washing with five column volumes of the same buffer. Elution was performed with a linear gradient of 30–0% ammonium sulfate saturation in buffer A. Active fractions were collected and concentrated by ultrafiltration using Amicon ultra-15 (Merck Millipore) for the final step. The concentrated enzyme solution (<1 mL) was applied to a ProteoSEC Dynamic 6-600kDa HR resin (Protein Ark) column previously equilibrated with buffer A containing 0.15 M NaCl. The column was washed with the same buffer, and the active fractions were saved as the purified enzyme preparation.

The recombinant proteins, wild-type BoGAPC, C156S, C160S, and C156S/C160S were purified using the Ni–NTA column and Sephacryl S-100 as previously described [20].

Se quantification

Se concentrations were measured using Hydride Generated-Atomic Fluorescence Spectrometry (HG-AFS: Millennium Excalibur, PSA, Orpington, UK). Measurement conditions were as follows: flow rate, 1 mL/min; injection volume, 100 µL; acid carrier, 50% (v/v) HCl; reductant, and 0.7% (w/v) $NaBH_4$ in 0.1% NaOH. Se concentrations were assessed based on a calibration curve created using selenate.

Synthesis of Se-binding GAPC (Se-GAPC) *in vitro*

Purified GAPC was reduced by DTT and reacted with a reaction mixture containing GSSeSG or selenite at room temperature for 10 min. GAPC after the reaction was separated into GAPC and a low-molecular-weight Se compound using the PD-10 column (GE Healthcare). Eluted fractions were applied to Se measurements by HG-AFS and protein quantification by the UV absorption method at 280 nm, and the selenylation of GAPC was evaluated. GSSeSG solution was prepared by reacting selenite and GSH at a concentration ratio of 1:4 at 24°C for 10 min in 50 mM potassium phosphate (pH 7.4).

Detection of selenol groups with BES-Thio

A 25 µM 3'-(2,4-dinitrobenzenesulfonyl)-2',7'-dimethylfluorescein (BES-Thio, Wako) solution was prepared by dissolving BES-Thio in 99.5% ethanol and diluting it with 50 mM potassium phosphate buffer at pH 5.8. BES-Thio solution was mixed with the sample solution to a final BES-Thio concentration of 22.5 µM, and then incubated at 37°C for 10 min. Fluorescence intensity was measured using a fluorometer (Excitation: 495 nm, Emission: 535 nm).

Results

Purification and Se quantification of GAPC

To examine the synthesis of Se-GAPC and its enzymatic properties *in planta*, we selected the appropriate Se concentration to add to *B. oleracea* during cultivation. The effects on fresh weight and hypocotyl length revealed that selenate at concentrations of 5 µM or higher had a negative effect on the plants (**Fig. S1**). On the other hand, plants supplemented with 1 µM selenate showed no growth inhibition and maintained high GAPC activity compared to the control plants. Therefore, the optimal concentration of selenate to be added during cultivation was determined to be 1 µM to obtain Se-supplemented plants for subsequent experiments. Furthermore, GAPC activity was significantly stronger than that of the control. To elucidate the cause of enhanced GAPC activity following the addition of selenate, GAPC was purified from control and 1 µM selenate-supplemented plants. The results of SDS-

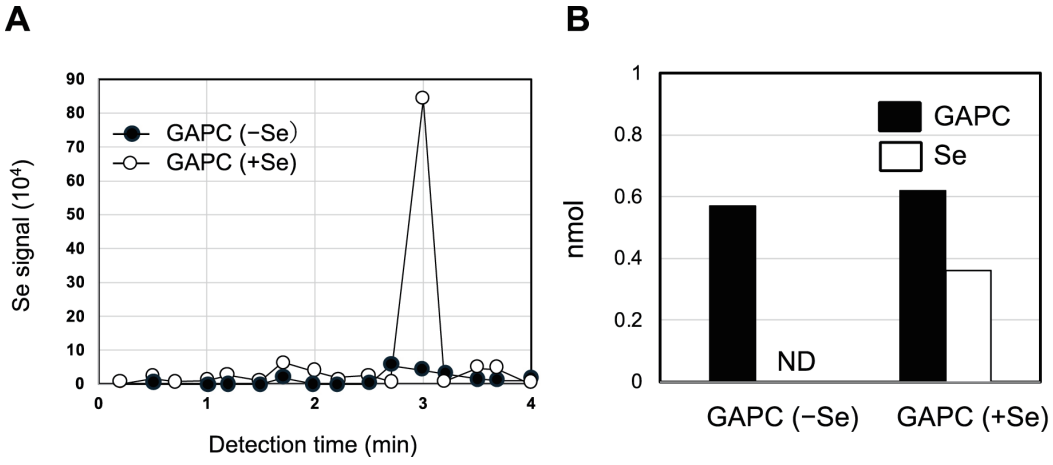


Fig. 1. **Analysis of selenium in purified GAPC(-Se) and GAPC(+Se).**
(A) Detection of selenium in purified GAPC(-Se) and GAPC(+Se) using HG-AFS. Detected fluorescence was applied to the calibration curve prepared from signals of selenite with varying concentrations to calculate the amount of selenium in the sample. (B) The relationship between relative quantities of selenium and purified GAPC. Quantities of purified GAPC were assessed by UV absorbance.

PAGE followed by the silver staining of both samples showed the presence of a single band at a molecular weight of approximately 37,000 Da in the GAPC active fraction by gel filtration chromatography (**Fig. S2**). GAPC active fractions from control and 1 μM selenate-supplemented plants were recovered as GAPC(-Se) and GAPC(+Se), respectively. Comparisons of the specific activities of each purified enzyme confirmed that GAPC(+Se) exhibited 1.37-fold stronger specific activity than GAPC(-Se) (**Table S1**). HG-AFS detected a fluorescence peak, indicating the presence of Se from GAPC(+Se), thereby proving the binding of Se to GAPC(+Se) with enhanced activity (**Fig. 1A**). The results of Se quantification by HG-AFS and protein quantification showed that 0.62 nmol of GAPC(+Se) contained 0.33 nmol Se (**Fig. 1B**).

Mechanisms underlying Se-GAPC synthesis

The results of enzyme activity measurements and Se quantification using purified GAPC demonstrated that Se-GAPC was synthesized *in planta*, which enhanced GAPC activity. Since previous studies reported that Se binding by PTMs in proteins involved GSH *in vivo*, we focused on GSH and investigated the mechanisms underlying the synthesis of Se-GAPC. Using buthionine sulfoximine (BSO), which inhibits GSH biosynthesis, we cultivated GSH-limited plants. The results obtained showed that the addition of 10 μM BSO reduced the total GSH level of both control and 1 μM selenate-supplemented plants to 30% of that without BSO (**Fig. S3A**). Comparisons of GAPC activity between GSH-unrestricted and -restricted plants showed that Se-induced enhancements in GAPC activity were not observed in GSH-restricted plants (**Fig. S3B**). We then attempted to synthesize Se-GAPC *in vitro* for a more detailed investigation, including the enzymatic properties of Se-GAPC. This method was designed based on the result showing that Se binding to catalytic Cys was caused by GSH-mediated PTM. After the reaction between GSSeSG, which was produced by GSH reducing selenite, and reduced GAPC, the mixed solution was applied to a desalting column (PD-10). As a result of separating the reaction mixture containing GAPC and GSSeSG, elution peaks of one GAPC and two Se were detected, and one of the two Se was eluted simultaneously with GAPC. The number of moles of Se eluted simultaneously with GAPC was four-fold that of GAPC (**Fig. 2A**). Since the PD-10 elution pattern of GSSeSG solution only showed one Se peak between elution volumes of 7-11 mL (**Fig. 2B**), the Se elution peak was not consistent with that of GAPC between elution volumes of 7-11 mL in **Fig. 2A**, indicating that low-molecular-weight Se did not bind to GAPC. Based on these results, the reaction between reduced GAPC and GSSeSG generated Se-GAPC, which exhibited 1.6-fold stronger GAPC activity than that of GAPC (**Fig. 2C**).

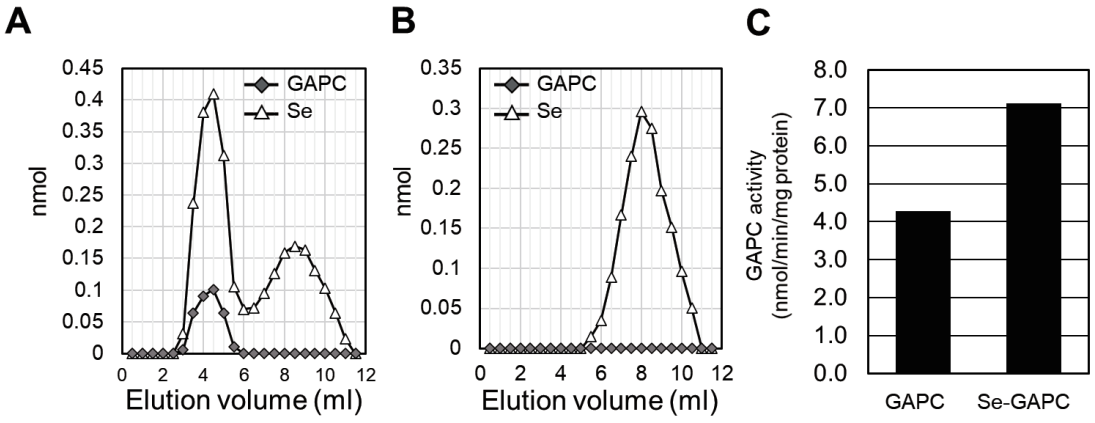


Fig. 2. **Synthesis of Se-GAPC by the reaction between GAPC and GSSeSG *in vitro*. Separation results of the reaction solution containing GAPC and GSSeSG (A) or only GSSeSG (B).**
Separation of the solution was performed using PD-10, with high molecular weights (>5000 Da) and low molecular weights (<1000 Da) being separated into fractions. The amounts of Se and proteins were analyzed by HG-AFS and UV absorption at 280 nm, respectively. (C) Comparison of the enzyme-specific activities of Se-GAPC and GAPC.

Analysis of enzyme properties using Se-GAPC synthesized *in vitro*

Following the successful synthesis of Se-GAPC by the reaction between reduced GAPC and GSSeSG *in vitro*, we conducted a detailed analysis of the properties of Se-GAPC. We compared the fluorescence of BES-Thio to those of GAPC and Se-GAPC at pH 5.8 to prove the hypothesis that the enhanced enzymatic activity of Se-GAPC is due to the conversion of the thiol group (SH) of catalytic Cys to a selenol group (SeH) by Se bonds. BES-Thio fluoresces when deprotected by aromatic nucleophilic substitution with a thiol or selenol group because the pK_a(SeH) of Sec is 5.2, which is markedly lower than the pK_a(SH) of 8.3 for the thiol cysteine (Cys), and selenol acts as a stronger nucleophile than the thiol. **Fig. 3A** shows the results of a preliminary experiment using Cys and Sec as models for the thiol and selenol groups; at pH 5.8, the fluorescence intensity of BES-Thio was stronger for Sec than for Cys. In the experiment using GAPC and Se-GAPC, the same results were obtained for Se-GAPC as those shown in **Fig. 3A;** fluorescence intensity at pH 5.8 was stronger than that of GAPC, which demonstrated that Se formed a selenol group in Se-GAPC (**Fig. 3B**). Reduced and purified recombinant GAPC (BoGAPC) and mutants (C156S, C160S, and C156S/C160S) were then reacted with GSSeSG to confirm the binding of Se to BoGAPC and mutant proteins and

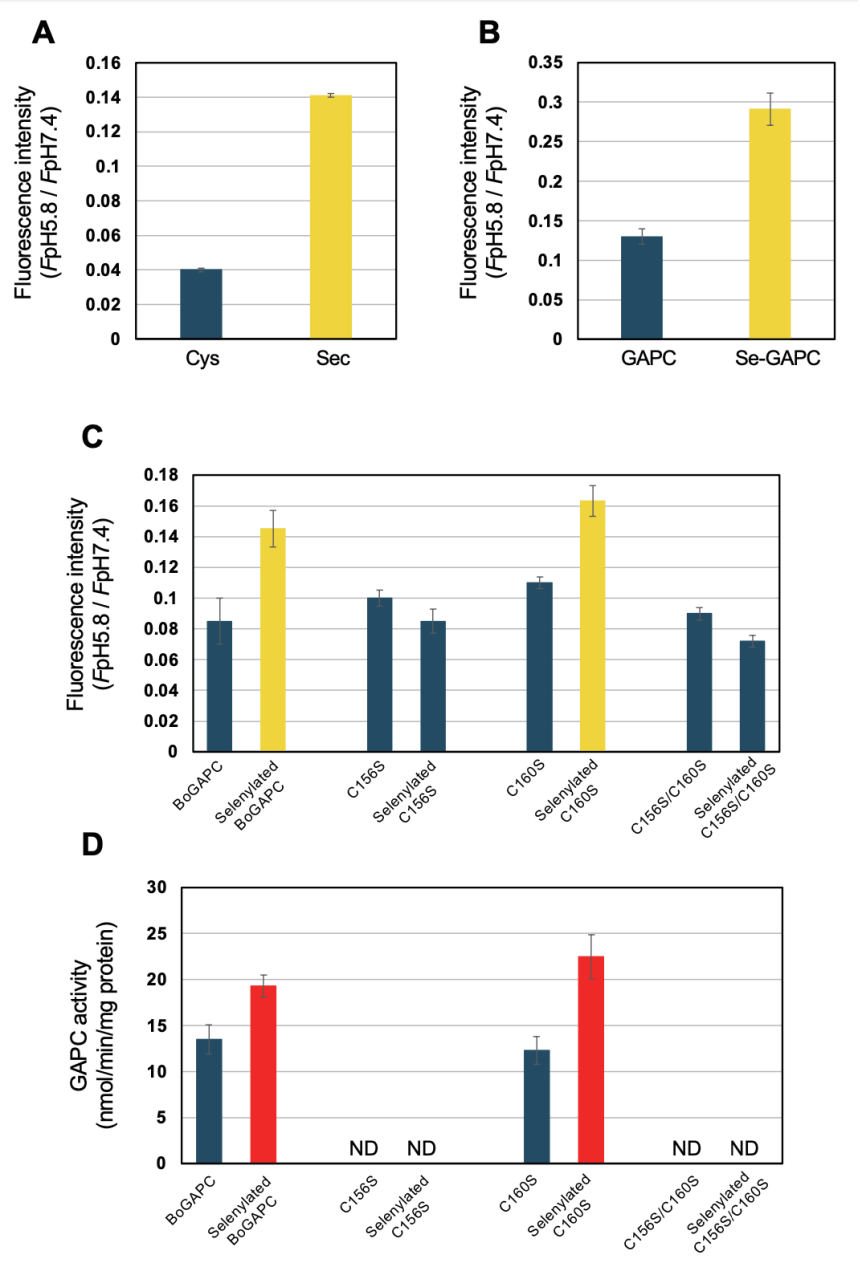
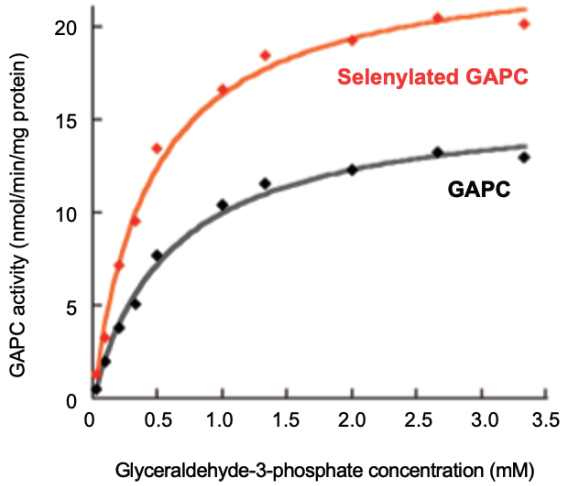


Fig. 3. **Selenol group generation in GAPC from *B. oleracea* and recombinant GAPC (BoGAPC and mutants) depending on selenylation.** Fluorescence intensity from the reaction of each sample with BES-Thio at pH 5.8. (A) Cys(-SH) and Sec(-SeH). (B)GAPC and Se-GAPC. (C) BoGAPC and mutants (C156S, C160S, and the double mutant C156S/C160S). (D) GAPC activity of BoGAPC and mutants (C156S, C160S, and the double mutant C156S/C160S). ND means not detected. Values represent the means ± SD of three experiments.

the generation of selenol groups by PTM. At the same time, the activity of GAPC was measured. No selenol group was detected in the mutant (C156S) in which catalytic Cys was mutated to Ser or in the double mutant (C156S/C160S), and there was no enzyme activity. In contrast, BoGAPC and C160S, in which Cys 156 was conserved, possessed a selenol group and increased enzyme activity, respectively (**Fig. 3C, D**). These results indicate that GAPC with a selenol group at catalytic Cys (selenylated GAPC) exhibited stronger nucleophilic reactivity than GAPC due to the mechanism of action of BES-Thio, which supports enhancements in enzyme activity by Se binding. To investigate the effects of Se binding to catalytic Cys on the activity of GAPC, we performed GAPC activity assays using selenylated GAPC synthesized *in vitro* with BoGAPC. Based on the results obtained, the effects of the selenylation of GAPC were examined by comparing the enzyme properties of GAPC and selenylated GAPC. As shown in **Fig. 4**, two enzyme parameters, K_{cat} and catalytic efficiency (K_{cat}/K_m), showed that selenylated GAPC catalyzed G3P more efficiently than GAPC. The V_{max}, K_{cat} and K_{cat}/K_m values of selenylated GAPC were 1.49-, 1.48- and 1.86-fold higher, respectively, than those of GAPC. These results indicate that the generation of a selenol group to the catalytic Cys of GAPC by PTM increased enzyme activity.

A



B

	GAPC	Selenylated GAPC
K_m	0.60	0.45
V_{max}	16.0	23.8
k_{cat}	2.37 min^{-1}	3.51 min^{-1}
k_{cat}/K_m	0.07 $M^{-1}sec^{-1}$	0.13 $M^{-1}sec^{-1}$

Fig. 4. **Michaelis-Menten plot of kinetics of GAPC and selenylated GAPC on glyceraldehyde-3-phosphate as the substrate (A). Kinetic characterization of GAPC and selenylated GAPC (B).**
Data are shown as the mean value of triplicate measurements.

Discussion

The selenylation of GAPC was synthesized in *B. oleracea var. italica* grown in the medium containing 1 μM selenate

GAPC(+Se) purified from 1 μM selenate-supplemented plants exhibited stronger enzyme-specific activity and Se binding of 0.5 to 1 mol of GAPC than GAPC(-Se) from control plants. This result confirmed enhancements in enzyme activity by Se binding to proteins, which is consistent with previous findings from *A. thaliana* and *C. reinhardtii* [11, 12]. On the other hand, since GAPC forms homotetramers and has four catalytic Cys sites per molecule, 4 mol of Se is expected to bind to 1 mol of GAPC. However, GAPC(+Se) showed binding of 0.5 mol of Se to 1 mol of GAPC, which was lower than expected. Previous studies using rhodanese, in which Se binding to catalytic Cys in the active center was confirmed, reported that Se was released from rhodanese following the treatment of Se-bound rhodanese with DTT [16]. Therefore, a certain amount of Se is detached from GAPC(+Se) during the process of GAPC purification using a buffer containing DTT, which contributes to maintaining the stability of proteins with catalytic Cys. Although the exact amount of Se binding in GAPC (+Se) before purification remains unknown, it may be higher than the value obtained in the present study.

The reaction by GSSeSG converts GAPC to selenylated GAPC

The results of experiments using GSH-restricted plants suggest the involvement of GSH in the selenylation of Se-GAPC *in planta*. GSH is a biomolecule that plays a role in the maintenance of the cellular redox balance in many

species, including plants, and reduces selenite to GSSeSG, the glutathione selenide anion ($GSSe^-$), and HSe^- by non-enzymatic reactions [21]. Selenylated GAPC, in which GAPC and Se are bound at a molar ratio of 1:4, was recovered from the *in vitro* reaction of GSSeSG with reduced GAPC. Since GAPC is a homotetramer-forming protein with one catalytic Cys site per subunit, it is reasonable that a 4-fold higher amount of Se than GAPC was recovered. These results clearly demonstrate the role of GSH in Se binding by PTMs to the catalytic Cys of GAPC. However, since GSSeSG is unstable in neutral solution and rapidly decomposes into GSSe- and HSe-, GSSeSG may not be directly involved in the transition of Se to catalytic Cys in the protein. Previous studies on the S-sulfhydration of proteins demonstrated that the gaseous compound sulfide (H_2S) functioned as a sulfur donor to catalytic Cys [22]. Based on these findings, we speculate that HSe- generated by the decomposition of GSSeSG is the direct Se donor to the catalytic Cys. Se binding to Cys in GAPC in plant cells, however, occurs under reducing conditions, not oxidizing conditions. Therefore, as proposed by Ogasawara et al. [16], it is highly likely that Cys in GAPC undergoes thiol exchange with GSSeSG to form Cys-S-Se-SG, which is then reduced by physiological reducing agents such as glutaredoxin and thioredoxin to generate Cys-S-SeH.

Selenylation of GAPC and enhancements in enzyme activity

Experiments using BES-Thio showed that GSSeSG converted the thiol group of the catalytic Cys to a selenol group, resulting in an increase in the nucleophilic reactivity of the catalytic site of selenylated GAPC. Based on previous findings, we speculated that the selenol group of selenylated GAPC was due to S-selenylcysteine (Cys-S-SeH). Se binding to the catalytic Cys of GAPDH, a non-selenoprotein, by a bond similar to the disulfide bond cleaved by DTT was previously confirmed in *E. coli* [23]. The release of Se bound to Cys residues in proteins by a treatment with DTT has also been reported in Se-binding rhodanese[16].

Physiological significance other than enhancements in enzyme activity *in planta*

Previous studies on S-sulfhydration revealed more than 2000 different S-sulfhydrated proteins in *A. thaliana* [22]. Furthermore, S-sulfhydration positively and negatively affected enzyme activities: GAPDH, APX, and ParkinE3 ligase were activated by a NaHS treatment [22,24], while PTP1B enzyme activity was inactivated [25]. Therefore, Se binding to the catalytic Cys of proteins, i.e., S-selenylation, is expected to occur in proteins other than GAPC, resulting in enhanced or inactivated enzyme activity. S-sulfhydration also functions as a stress signaling mechanism by regulating the subcellular localization of various proteins, including GAPC [26-28]. Based on these findings, we hypothesize that the S-selenylation of proteins, including GAPC, is involved in intracellular signaling and may be one of the unidentified Se dynamics in plants.

Furthermore, S-selenylation of GAPC in plant cells maintained in reducing state under non-stress conditions is thought to be one of the mechanisms to avoid damage caused by highly toxic Se compounds, such as selenate randomly taken up via sulfate transporters in the roots, and selenite and Sec produced from selenate via the dissimilatory reduction pathway.

Supplementary data

Table S1. Purification of GAPC from *Brassica oleracea var.italica* grown in MS medium with or without 1 µM selenate. Control refers to plants grown in MS medium that does not contain selenate.

Control

	Total protein (mg)	Total activity (µmol/min)	Specific activity (µmol/min/mg protein)	Yield (%)	Purification rate (fold)
Crude extract	91.4	2.41	0.026	100	1.0
Ultra Centrifugation	76.4	2.08	0.027	86.3	1.0
30% ammonium Sulfate Precipitation	48.4	1.30	0.027	53.9	1.0
Hydrophobic Interaction Chromatography	0.94	0.18	0.19	7.47	7.31
Gel Filtration Chromatography	0.008	0.033	4.13	1.37	158.8

1 µM Selenate

	Total protein (mg)	Total activity (µmol/min)	Specific activity (µmol/min/mg protein)	Yield (%)	Purification rate (fold)
Crude extract	50.3	1.48	0.029	100	1.0
Ultra Centrifugation	42.6	1.24	0.029	83.8	1.0
30% ammonium Sulfate Precipitation	27.5	0.88	0.032	59.5	1.1
Hydrophobic Interaction Chromatography	1.22	0.21	0.17	14.2	5.86
Gel Filtration Chromatography	0.007	0.04	5.71	2.70	196.9

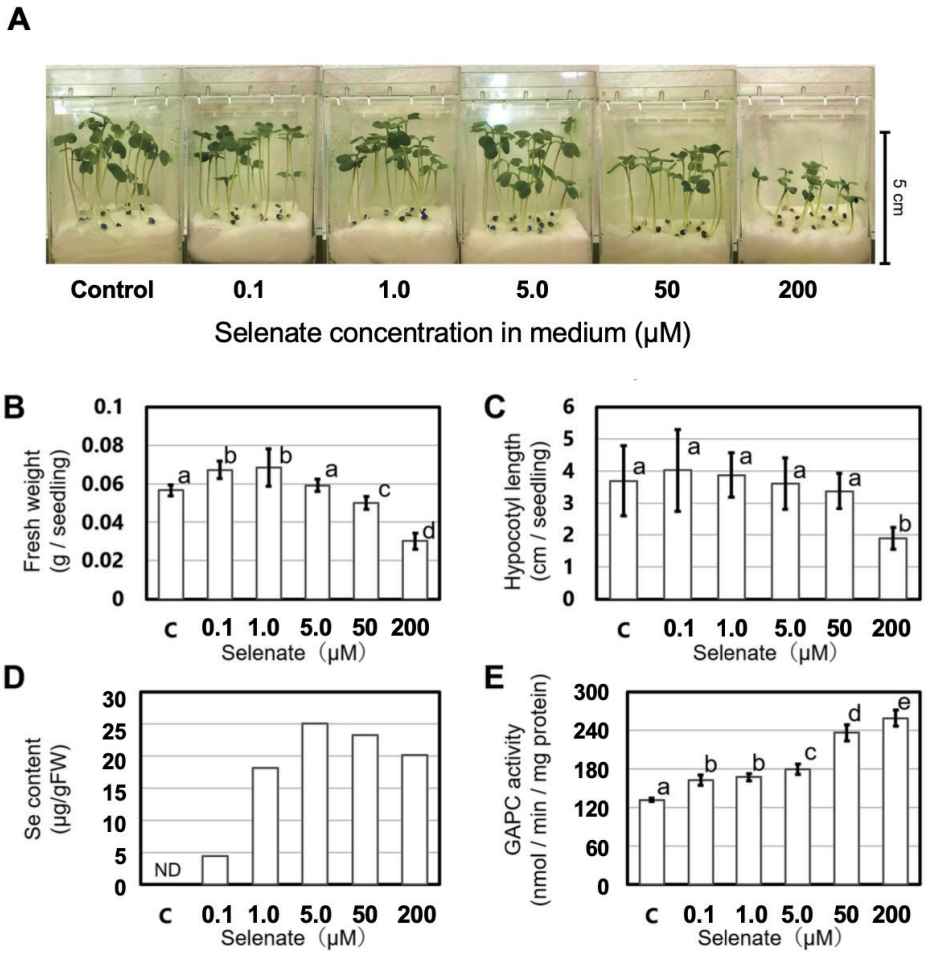


Fig. S1. **Effects of selenate on the growth of *Brassica oleracea var.italica*.**
(A) Growth of plants with MS medium containing 0-200 μM selenate. Control refers to no addition of selenate. (B) Fresh weight as a growth parameter. (n=20). (C) Hypocotyl length as a growth parameter. (n=20). (D) Results on the Se quantification of crude extracts showing the incorporation of Se into plants. (E) GAPC activity in crude extracts prepared from plants grown in various concentrations of selenate (n=3). Different letters represent a significant difference ($p < 0.05$).

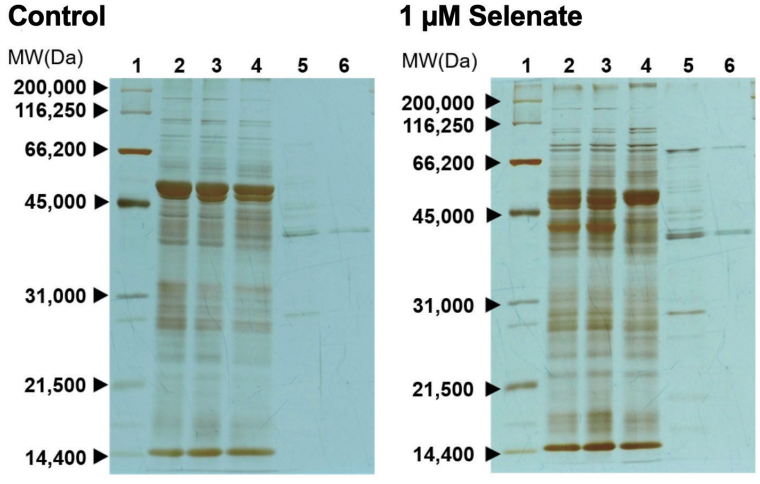


Fig. S2. **Results of silver staining following SDS-PAGE.**
The numbers at the top of the lane indicate the respective applied samples. (1) Protein marker (2) Crude extract (3) Ultra centrifugation (4) 30% ammonium sulfate precipitation (5) Hydrophobic interaction chromatography (6) Gel filtration chromatography

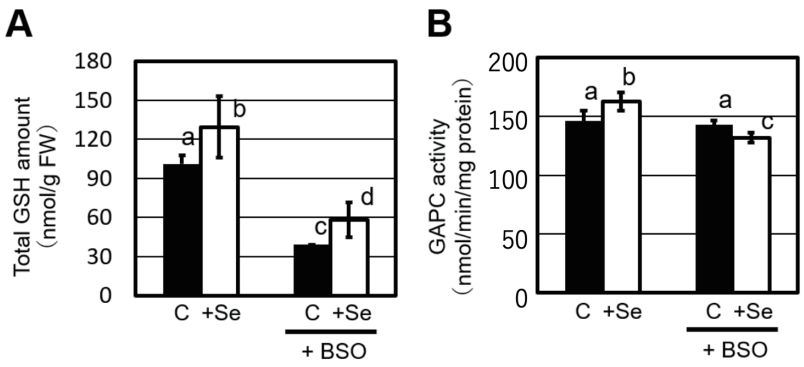


Fig. S3. **Effects of GSH on Se-binding GAPC synthesis *in planta*.**
(A) The amount of GSH in *B. oleracea* (control) and 1 μM selenate-supplemented plants (+Se) and the amount of GSH in plants cultivated in the presence of BSO. GSH-restricted plants were produced by addition of 10 μM BSO to control and 1 μM selenate-supplemented plants. (B) Effects of the restriction of GSH on GAPC activity in control and 1 μM selenate-supplemented plants (n=3). Different letters represent a significant difference ($p < 0.05$).

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Data Statement
Data that support the results of the present study are available from the corresponding author, T. T., upon reasonable request.

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