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Cytokinin is involved in *TPS22*-mediated selenium tolerance in *Arabidopsis thaliana*

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• **Background and Aims** Excess selenium (Se) is toxic to plants, but relatively little is known about the regulatory mechanism of plant Se tolerance. This study explored the role of the *TPS22* gene in Se tolerance in *Arabidopsis thaliana*.
• **Methods** Arabidopsis wild type and XVE mutant seeds were grown on half-strength MS media containing Na_2SeO_3 for screening of the Se-tolerant mutant *tps22*. The XVE T-DNA-tagged genomic sequence in *tps22* was identified by TAIL-PCR. The *TPS22* gene was transformed into the mutant *tps22* and wild type plants using the flower infiltration method. Wild type, *tps22* mutant and transgenic seedlings were cultivated on vertical plates for phenotype analysis, physiological index measurement and gene expression analysis.
• **Key Results** We identified an Arabidopsis Se-tolerant mutant *tps22* from the XVE pool lines, and cloned the gene which encodes the terpenoid synthase (*TPS22*). *TPS22* was downregulated by Se stress, and loss-of-function of *TPS22* resulted in decreased Se accumulation and enhanced Se tolerance; by contrast, overexpression of *TPS22* showed similar traits to the wild type under Se stress. Further analysis revealed that *TPS22* mediated Se tolerance through reduction of Se uptake and activation of metabolism detoxification, which decreased transcription of high-affinity transporters *PHT1;1*, *PHT1;8* and *PHT1;9* and significantly increased transcription of selenocysteine methyltransferase (SMT), respectively. Moreover, loss-of-function of *TPS22* resulted in reduced cytokinin level and repression of cytokinin signalling components *AHK3* and *AHK4*, and upregulation of *ARR3*, *ARR15* and *ARR16*. Exogenous cytokinin increased transcription of *PHT1;1*, *PHT2;1* and *SMT* and decreased Se tolerance of the *tps22* mutant. In addition, enhanced Se resistance of the *tps22* mutant was associated with glutathione (GSH).
• **Conclusions** Se stress downregulated *TPS22*, which reduced endogenous cytokinin level, and then affected the key factors of Se uptake and metabolism detoxification. This cascade of events resulted in reduced Se accumulation and enhanced Se tolerance.

Key words: *TPS22*, selenium tolerance, cytokinin, selenium uptake, selenium detoxification, *Arabidopsis thaliana*.

INTRODUCTION

Selenium (Se) is a micronutrient for many organisms but is also toxic at higher levels, and both Se deficiency and Se toxicity are problems worldwide (Pilon-Smits, 2015; Pilon-Smits *et al.*, 2017). Selenate and selenite are the predominant form of Se in soils, and plants absorb them by different mechanisms (Zhu *et al.*, 2009; Huang *et al.*, 2015). Selenate is absorbed by the sulfate transporter, and is metabolized through the sulfur (S) assimilation pathway due to the similar chemical properties between Se and S (Terry *et al.*, 2000; Cabannes *et al.*, 2011; Liu *et al.*, 2016). Selenite is absorbed by the phosphate (Pi) transporters (Li *et al.*, 2008; Zhang *et al.*, 2014; Schiavon and Pilon-Smits, 2017). In Arabidopsis, at least five phylogenetically distinct classes of integral membrane proteins possess Pi transport activity. The *PHT1* genes, *PHT1;1–PHT1;9*, encode high-affinity plasma membrane-localized Pi transporters, which mediate Pi import across the plasma membrane and catalyse Pi/H^+ symport activity. All *PHT1* genes, except *PHT1;6*, are predominantly expressed in root tissues. They mediate external Pi uptake, which represents the primary and crucial step in plant Pi acquisition (Remy *et al.*, 2012). The *PHT2;1* gene encodes a low-affinity Pi transporter located in the chloroplast inner envelope membrane that mediates Pi translocation within the aerial parts of the plant and influences whole-plant Pi allocation (Daram *et al.*, 1999; Versaw and Harrison, 2002; Remy *et al.*, 2012). Selenite is absorbed by roots and then converted to organic Se to transport to the shoots. In the chloroplast, selenate is reduced to selenite, and then through a variety of complex chemical reactions to generate selenocysteine (SeCys), selenomethionine (SeMet) and other forms of organic Se (Gabel-Jensen & Gammelgaard, 2010; Banuelos *et al.*, 2011; Pilon-Smits, 2015). Se toxicity in plants has been attributed to the formation of non-specific selenoproteins and oxidative stress (Van Hoewyk, 2013). When seleno amino acids inadvertently become incorporated into proteins, replacing Cys and Met, this impairs protein function and thus results in toxicity (Stadtman, 1990; Pilon-Smits, 2015; Gupta and Gupta, 2017).

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Se non-accumulator plants which accumulate less than 100 mg Se/kg d. wt, e.g. grasses and crops, can metabolize SeMet into volatile dimethylselenide (DMSe) (Terry *et al.*, 2000; Gupta and Gupta, 2017). DMSe was found to be almost 600 times less toxic than inorganic Se compounds (McConnell and Portman, 1952; Wilber, 1980). The production of DMSe is important for non-accumulators to divert potentially toxic SeMet to the significantly less toxic DMSe (Pilon-Smits *et al.*, 2017). Se hyperaccumulator plants accumulate more than 1000 mg Se/kg d. wt, e.g. *Astragalus* and *Stanleya*, and thrive well in Se-rich soil (Pilon-Smits *et al.*, 2017). They have methylated forms of SeCys and SeMet. SeCys is methylated to form MetSeCys by selenocysteine methyltransferase (SMT), and finally becomes volatile dimethyldiselenide (DMDSe) (Sors *et al.*, 2005; Pilon-Smits, 2015). DMSe is the main volatile Se compound in non-accumulators, while DMDSe is primarily produced in hyperaccumulators (Pilon-Smits and Le Duc, 2009; Pilon-Smits *et al.*, 2017). Aside from volatilization, another Se detoxification mechanism in plants is the breakdown of SeCys into elemental Se and alanine by selenocysteine lyase (SL) (Van Hoewyk *et al.*, 2005; Van Hoewyk, 2013; Pilon-Smits, 2015). Selenocysteine lyases are analogous to NifS-like Cys desulfurase proteins characterized in Arabidopsis (Pilon-Smits *et al.*, 2002; Ye *et al.*, 2005). The overexpression of *AtCpNifS* can enhance Se tolerance and accumulation in Arabidopsis (Van Hoewyk *et al.*, 2005; Van Hoewyk, 2013).

Molecular and biochemical studies of non-accumulator plants revealed that plant hormones play an important role in Se defence responses. It was found that genes involved in the ethylene and jasmonic acid pathways were up-regulated by Se in Arabidopsis (Tamaoki *et al*., 2008*a*, *b*). These phytohormones are enhanced via signal pathways of reactive oxygen species (ROS) and then act in a cooperative or antagonistic manner to induce stress responsive genes and Se uptake and metabolic genes (Pilon-Smits *et al.*, 2017; Schiavon and Pilon-Smits, 2017). Previous research also proposed that selenite-induced H_2O_2 mitigates a selenite-defensive response (Tamaoki *et al.*, 2008*b*; Lehotai *et al.*, 2012; Van Hoewyk, 2013; Jiang *et al.*, 2016). In addition, Se affects cytokinin (CK) levels in primary roots (Pilon-Smits *et al.*, 2017), while nitric oxide (NO) represses CK signalling (Feng *et al.* 2013). The higher levels of NO improved Se tolerance in the Arabidopsis mutant *gsnor1-3* (Lehotai *et al*., 2011, 2012).

Some key genes involved in Se tolerance have been cloned and analysed. The overexpression of ATP sulfurylase leads to selenate tolerance (Pilon-Smits *et al.*, 1999). The overexpression of the Se binding protein gene (*SBP1*) in Arabidopsis can increase resistance to selenite (Agalou *et al.*, 2005; Hugouvieux *et al.*, 2009). However, our understanding of the molecular mechanisms of Se tolerance is far from complete. In this study, we identified an Arabidopsis Se-tolerant mutant *tps22* from *XVE*-tagged T-DNA insertion lines (Zhang *et al.*, 2005) and cloned the corresponding gene which encodes the terpenoid synthase (*TPS22*). In plants, terpene synthases (TPSs) are responsible for the synthesis of a large class of terpene compounds consisting of a five-carbon isoprene-building unit. Some hormones, for example CKs, are made up by terpenes (Chen *et al.*, 2011; Falara *et al.*, 2011; Tholl and Lee, 2011). Our study demonstrated that CK is involved in *TPS22*-medaited Se tolerance and aids in the search for the mechanism of Se detoxification and tolerance in plants.

MATERIALS AND METHODS

Plant materials, growth conditions and treatments

The plant materials used in this study included wild-type (WT) *Arabidopsis thaliana* (L.) Heynh Columbia-0 (Col-0), *tps22* mutant, transgenic plants (*TPS22*-complementary plant COM1; *TPS22*-overexpressing plant OE1) and *ipt1 3 5 7* mutant (Miyawaki *et al.*, 2006). The *tps22* mutant was screened from the XVE-tagged T-DNA insertion lines (Zhang *et al.*, 2005). Seeds of the *ipt1 3 5 7* quadruple mutant were kindly provided by Dr Dongwei Di.

For phenotype analysis, seeds of WT, mutant or transgenic plants were germinated and grown vertically on half-strength (½) MS (Murashige and Skoog, 1962) media, sodium selenite (Na_2SeO_3), β-oestradiol (ES), 6-benzylaminopurine (BA) or buthionine sulfoximine (BSO; Sigma). The plates were stored for 3 d in the dark at 4°°C and then placed in a growth chamber maintained at 22 °C and 65 % humidity with a light intensity of 100 μmol m^{-2} s^{-1} and a 16-h day length. After the indicated days of growth, plants were sampled for root growth assays and measurement of fresh weight. In addition, the seeds were sown in pots of peat soil/vermiculite/perlite (3: 9: 0.5) presoaked with plant nutrient solution and grown at 22 °C and 65 % humidity with a light intensity of 100 μmol m^{-2} s^{-1} and a 16-h day length.

Wild-type, *tps22* mutant and transgenic plants were grown on ½ MS media with or without 30 μm Na_2SeO_3, 0.01 μm BA or 0.1 mM BSO. After the indicated days of growth, seedlings were sampled for Se, Pi, CK and GSH content, GPX activity, ROS level and gene expression analysis. To investigate the organ-specific RNA level of *TPS22*, the roots, rosette leaves, cauline leaves, stems, inflorescence and siliques were obtained from WT plants grown in soil for 5 weeks.

Screening, identification of mutant and genetic analysis

The *tps22* mutant was screened as previously reported (Jiang *et al.*, 2016). WT and XVE mutant seeds were grown on ½ MS media for 7 d and transferred to ½ MS media containing 10 μm β-oestradiol and 45 μm Na_2SeO_3. Putative mutant seedlings that had a longer root length were transferred to soil to produce seeds.

The XVE T-DNA-tagged genomic sequence in *tps22* was identified by TAIL-PCR (Liu *et al.*, 1995; Zuo *et al.*, 2000) and DNA sequencing. The *tps22* mutant was crossed with the WT Col-0, and the F_1 seedlings were allowed to self-pollinate. F_1 and F_2 seedlings were then scored for the mutation trait.

Generation of TPS22*-complementary and* TPS22*-overexpressing transgenic lines*

Total RNA was extracted from seedlings using Trizol reagent (Invitrogen) according to the manufacturer's protocol and used to synthesize cDNA. The amplification reactions of the *TPS22* gene were performed in 20-μL volumes containing 2 μL of cDNA, 0.4 μL of each primer, 10 μL of PCR SuperMix and 7.2 μL of ddH_2O. The PCR thermocycling programme used was as follows: initial denaturation of 10 min at 95 °C; followed by 36 cycles of 30 s at 94 °C, 30 s at 48 °C and 2 min at 72 °C;

and a final extension reaction of 10 min at 72 °C. *TPS22* was fused to pCAMBIA1301 using the *Bst*EII and *Nco*I site of the pCAMBIA1301 vector. The *35S::TPS22* construct was introduced into *Agrobacterium tumefaciens* strain GV3101, which was transformed into the mutant *tps22* and WT plants using the flower infiltration method (Clough and Bent, 1998). The transcripts of *TPS22*-complementary and *TPS22*-overexpressing lines were detected by semi-quantitative reverse transcriptase PCR (RT-PCR). Total RNA was extracted from 2-week-old seedlings grown on ½ MS with or without 30 μM Na_2SeO_3. The primers used are listed in Supplementary Data Table S1.

RNA extraction, RT-PCR and quantitative real-time RT-PCR (qRT-PCR) analysis

Total RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's protocol and used to synthesize cDNA. RT-PCR was performed as previously described (Jiang *et al.*, 2016). qRT-PCR was performed according to the instructions provided for the Bio-Rad iCycler iQ system (Bio-Rad), using platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) as previously described (Jiang *et al.*, 2016). The primers used are listed in Table S1. Each sample was quantified at least in triplicate and normalized using *ACTIN11* as an internal control. Significant differences between the samples were evaluated by Student's *t*-test using delta Ct values (Yuan *et al.*, 2006).

Measurement of Se content

WT, *tps22* mutant and transgenic plants were each rinsed with 1 mM $CaSO_4$ and deionized water three times to eliminate any external Se, and then sampled for determination of Se content according to the method described by Liu *et al.* (2016). Plant tissues were digested with 5 mL HNO_3 overnight and then the sample solutions were transferred into a 50-mL volumetric flask with 0.1 M HNO_3. The digested samples were diluted with 3 % HCl and analysed using an atomic fluorescence spectrometry detector (AFS-3100).

Measurement of total Pi content

WT and *tps22* mutant plants were rinsed in distilled water, blotted dry and ground in liquid nitrogen. The ground tissues were homogenized in 1 % glacial acetic acid and centrifuged at 12 000 *g* for 5 min. Aliquots of the supernatant solution were assayed for Pi content analysis. The total Pi content in the samples was quantified using a phosphomolybdate colorimetric assay as described previously (Ames, 1966; Versaw and Harrison, 2002).

Measurement of CK content

The extraction and purification of CK was similar to the method described by Sun *et al.* (2003). In brief, WT, *tps22* mutant and transgenic seedlings were ground with 80 % (v/v) methanol. The homogenate was centrifuged at 5000 *g* for 10 min and the supernatant was collected for passage through a SEP-pak C-18 cartridge to remove the pigments and other lipophilic impurities. The filtrate was collected for CK content analysis. The CK content was determined using a plant CK ELISA Kit (Shanghai Sangon Biotech) following the manufacturer's instructions.

Measurement of GSH content

WT and *tps22* mutant seedlings were ground in liquid nitrogen and extracted in 1 mL of 5 % trichloroacetic acid. The homogenate was centrifuged at 15 000 *g* for 10 min. The supernatant was collected. Glutathione was measured based on the glutathione reductase-dependent reduction of 5,50-dithiobis (2-nitro-benzoic acid) (DTNB), monitored at 412 nm (Noctor and Foyer, 1998). The reaction mixture containing 10 μL supernatant, 0.1 mL of 0.2 M Na_2HPO_4 (pH 7.5), 10 mM EDTA, 10 μL of 10 mM NADPH, 10 μL of 12 mM DTNB and 60 μL of water was incubated for 5 min, and the absorbance was recorded at 412 nm. GSH content was expressed in μmol g^{-1} f. wt.

Analysis of GPX activity

WT, *tps22* mutant and transgenic seedlings were ground in liquid nitrogen and extracted in 50 mM sodium phosphate buffer (pH 7.0), containing 1 mM EDTA and 2 % (w/v) polyvinylpyrrolidone. The ground tissues were centrifuged at 12 000 *g* for 10 min. The supernatant was collected for GPX activity analysis according to the method of Tappel *et al.* (1978). GPX activity was tested based on the decrease of NADPH at 340 nm. Enzyme activity *U* is defined as the amount of NADPH (μmol) reduced per minute.

Determination of ROS

WT and *tps22* mutant plants were grown on ½ MS media for 7 d and then transplanted in media supplemented with or without 30 μM Na_2SeO_3 for the indicated times (12, 24 h). ROS were detected using 2′,7′-dichlorofluorescin diacetate (H_2DCFDA, a fluorescent dye for ROS) assays according to the method of Xie *et al.* (2011). The details were described by Jiang *et al.* (2017). ROS were determinated by using a TCS-SP2 laser scanning confocal microscope (LSCM, Leica Lasertechnik). Fluorescence was expressed as relative fluorescence units using Leica Confocal Software 2.5.

Statistical analysis

Experimental data are given as the means ± s.e. of three replicated groups. Statistical significance is determined by ANOVA combined with a post hoc test, and significant differences ($P < 0.05$) are indicated by different lowercase letters.

RESULTS

Isolation of Se-resistance mutant

To screen for Se-resistance mutants, we completed a genetic screen (Jiang *et al.*, 2016) and isolated two putative mutants

from the XVE-tagged T-DNA insertion lines (Zhang *et al.*, 2005). One of the mutants was chosen for further analysis. To test their Se tolerance, WT and mutant seeds were germinated and grown vertically on ½ MS media with or without 30 μM Na_2SeO_3 and 10 μM β-oestradiol for 3 weeks. There were no significant differences between the WT and the mutant plants that were grown on ½ MS media; however, when 30 μM Na_2SeO_3 was added to the ½ MS media with or without 10 μM β-oestradiol, the mutant seedlings were more resistant to Se than WT seedlings (Fig. 1A). Quantitative analyses confirmed that the root length and fresh weight of the mutant were significantly ($P < 0.05$) higher than those of the WT grown on Se-containing media with or without 10 μM β-oestradiol (Fig. 1B, C). These results suggest that the increased tolerance to Se in the mutant is independent of β-oestradiol. In addition, there were no significant differences between the WT and the mutant under soil cultivation for different periods, except 8 weeks (Fig. S1).

Genetic analysis of the Se-resistance mutant showed that the F_1 plants all exhibited the WT phenotype (Fig. S2). The F_2 seedings that resulted from the crosses showed a 3: 1 segregation ratio of the mutant to the WT (Table 1) (χ^2 test, $P > 0.05$). These results indicate that the Se-resistance mutant is a monogenic recessive nuclear mutation.

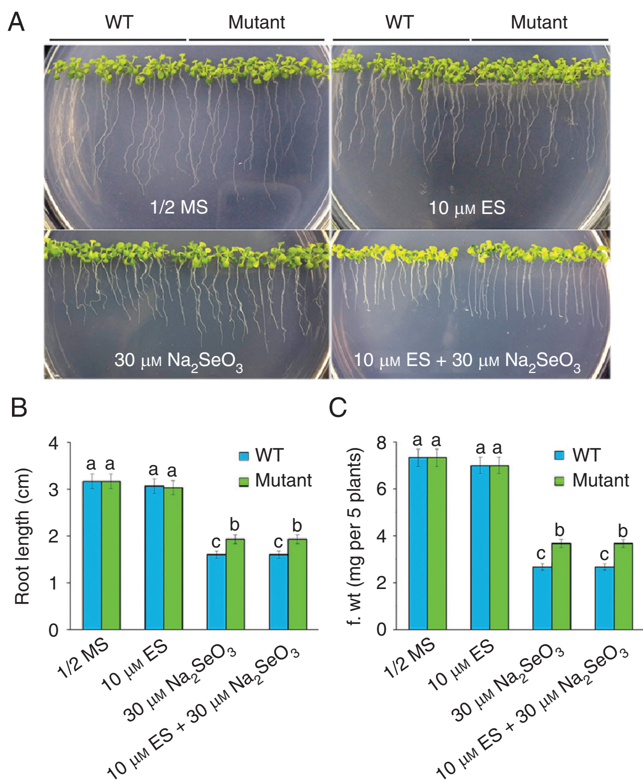

Fig. 1. Isolation of the Se-resistance mutant. (A) Arabidopsis wild-type (WT) and Se-resistance mutant plants grown on ½ MS media in the absence or presence of 10 μM β-oestradiol (ES) or 30 μM Na_2SeO_3 for 3 weeks. (B,C) Root length (B) and fresh weight (C) of the plants described in A. Data are the mean ± s.e. of three independent replicates. Significant differences ($P < 0.05$) are indicated by different lowercase letters.

Loss-of-function of TPS22 is responsible for the increased Se tolerance

We identified a single T-DNA insertion in the Se-resistance mutant using thermal asymmetric interlaced PCR (TAIL-PCR). The results indicate that T-DNA was inserted into the first exon downstream of the ATG start codon of the gene (*At1g33750*) (Fig. 2A), previously known as *TPS22* (Ro *et al.*, 2006). Therefore, this Se-resistance mutant was designated as *tps22*. We examined expression levels of the *TPS22* gene in the WT and *tps22* mutant. *TPS22* transcriptions in WT plants were detected and downregulated under Se stress, whereas transcription levels of *TPS22* were much lower (Fig. 2B) or not detected (Fig. 2C) in the *tps22* mutant in the absence or presence of Se. This suggests that the enhanced Se tolerance in *tps22* might be caused by loss-of-function of *TPS22*. The organ-specific expression pattern of *TPS22* by qRT-PCR analysis indicates that *TPS22* had a higher expression in the roots and a lower expression in the inflorescences and siliques (Fig. 2D).

To confirm that the enhanced Se tolerance in *tps22* was due to a loss-of-function mutant of *TPS22*, we generated transgenic *TPS22*-complementary and *TPS22*-overexpressing plants under the strong CaMV 35S promoter. Complement and overexpression of *TPS22* were verified by RT-PCR analysis (Fig. 2C). To test their Se tolerance, seeds of WT and transgenic plants were germinated and grown vertically on ½ MS media with or without 30 μM Na_2SeO_3 for 2 weeks. As shown in Fig. 2E, the complementary plants COM1 restored the growth state of the WT plants, and the OE1 plants showed similar traits to the WT plants. Quantitative analyses confirmed that the root length and the fresh weight of WT, COM1 and OE1 plants were similar and decreased more than those of the *tps22* mutant under Na_2SeO_3 (Fig. 2F, G).

TPS22 *mediates Se tolerance through reduction of Se uptake and activation of metabolism detoxification*

To test whether *TPS22*-mediated Se tolerance is associated with a change of Se content, we determined the Se content in WT, *tps22* and transgenic plants subjected to Se stress and found that Se content in *tps22* seedlings was significantly lower than that in the WT, COM1 and OE1 (Fig. 3A), suggesting that enhanced Se resistance in *tps22* might be associated with decreased Se content.

Previous research has shown that selenite uptake is mediated by phosphate transporters (Li *et al.*, 2008; Zhang *et al.*, 2014; Schiavon and Pilon-Smits, 2017). Therefore, we investigated the expression of Pi transporters *PHT1;1*, *PHT1;8*, *PHT1;9* and

Table 1. *Genetic analysis of the Se-resistance mutant*

Cross	Progeny	Total	Phenotype		χ^2
			+	–	
Mutant	F_1	30	30	0	
×Col-0	F_2	900	654	246	2.49

(+) wild-type; (–) mutant; χ^2 was calculated based on an expected ratio of 3: 1 (wild-type/mutant).

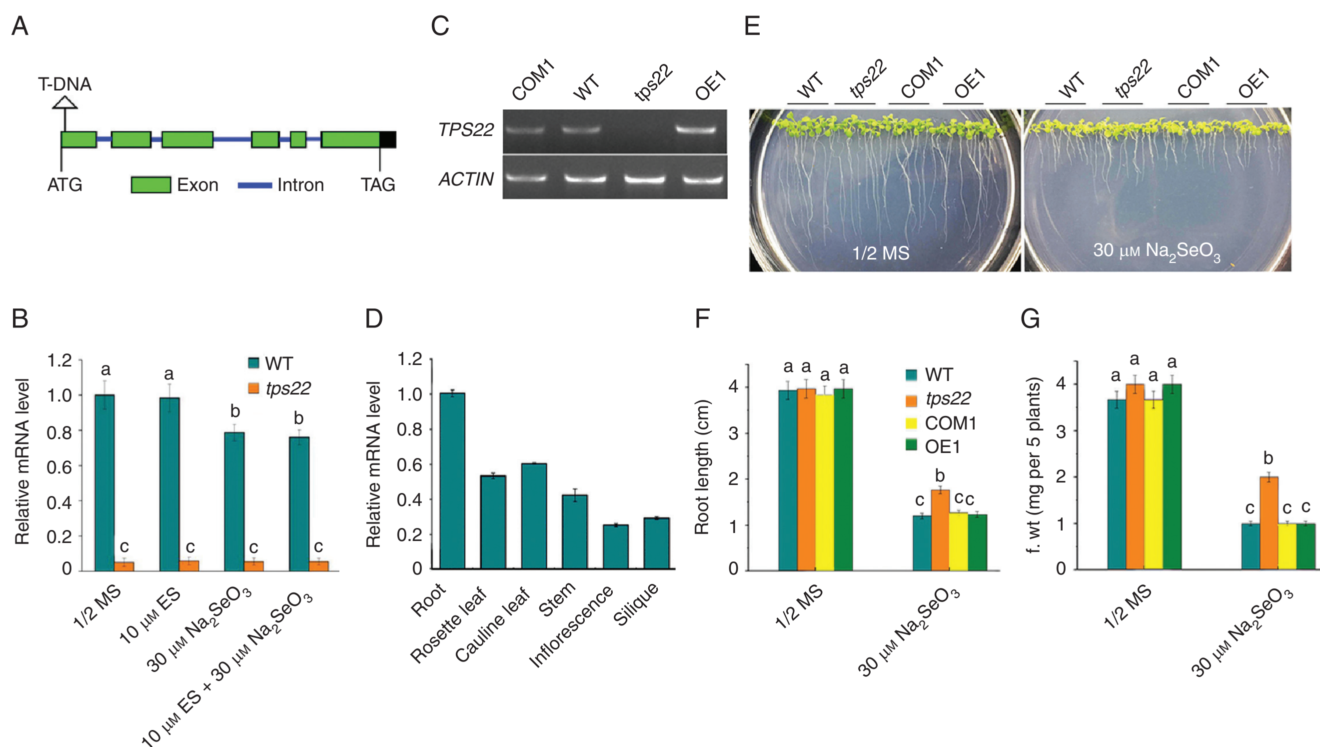

FIG. 2. Cloning of the *TPS22* gene. (A) A schematic of the T-DNA insertion site in TPS22. (B) qRT-PCR analysis of *TPS22* gene transcription levels in 2-week-old seedlings of the WT and *tps22*. *ACTIN11* was used as the internal control. (C) RT-PCR analysis of the *TPS22* gene transcriptions in 2-week-old seedlings of the WT, *tps22*, and *TPS22*-complementary (COM1) and *TPS22*-overexpressing (OE1) transgenic lines. *ACTIN11* (*ACTIN*) was used as a loading control. (D) qRT-PCR analysis of *TPS22* transcripts in different tissues of WT plants. RNA was isolated from roots, rosette leaves, cauline leaves, stems, inflorescence and siliques of the 5-week-old WT plants. *ACTIN11* was used as the internal control. (E) Phenotypes of WT, *tps22*, COM1 and OE1 plants grown on ½ MS media with or without 30 μM Na_2SeO_3 for 2 weeks. (F, G) Root length (F) and fresh weight (G) of the plants described in E. Data are the mean ± s.e. of three independent replicates. Significant differences ($P < 0.05$) are indicated by different lowercase letters.

PHT2;1 in WT, *tps22* and transgenic seedlings under Se stress. *PHT1;1* transcription level in the *tps22* mutant was significantly lower than that in WT in the absence or presence of Na_2SeO_3 (Fig. 3B). No significant differences in transcription levels of *PHT1;8*, *PHT1;9* and *PHT2;1* were observed between the WT and the *tps22* mutant grown on ½ MS media, but significantly lower transcription levels of these transporters were detected in the *tps22* mutant than in the WT under 30 μM Na_2SeO_3 stress, and transgenic lines were similar to WT (Fig. 3C–E). In addition, we measured total Pi content in WT and *tps22* mutant plants under Se stress, and the result showed Pi content in the *tps22* mutant was lower (Fig. S3). These results demonstrate that the lower expression level of these transporters reduces Se uptake and accumulation to contribute to Se tolerance.

SMT plays a crucial role in Se detoxification and its high efficiency in methylation of selenocysteine prevents the non-specific incorporation of selenoamino acids into proteins (Lyi *et al.*, 2005; Cakir and Ari, 2013). SL metabolizes selenocysteine into Se and alanine. Here, we detected significantly higher transcription levels of *SMT* in *tps22* (Fig. 4A), while the transcription level of SL was less reduced in the *tps22* mutant than in the WT treated with 30 μM Na_2SeO_3 (Fig. 4B), suggesting that the *tps22* mutant has a stronger ability to remove the toxic effect of Se.

The *GPX1* gene encodes Se-dependent glutathione peroxidase (GPX), an ROS scavenger in plants (Stadtman, 1990; Van Hoewyk, 2013). A significantly higher transcript level of *GPX1* was detected in *tps22* plants than in WT plants under Se stress (Fig. 4C). GPX activity of the *tps22* mutant improved more compared to that of the WT and transgenic lines (Fig. 4D). In addition, we also measured ROS accumulation in the WT and mutant plants under Na_2SeO_3 stress. A significantly lower increase of fluorescence was detected in *tps22* plants under Na_2SeO_3 treatment for 12 h, especially for 24 h (Fig. 5), in agreement with the higher level of *GPX1* and GPX activity of the *tps22* mutant.

Together, the above results suggest that *TPS22* mediates Se tolerance through reduction of Se uptake and activation of metabolism detoxification.

Cytokinin is involved in the regulation of TPS22*-mediated Se tolerance*

CKs are mainly synthesized in roots and made up by terpenes which are synthesized by TPSs, including TPS22 (Kamada-Nobusada and Sakakibara, 2009; Falara *et al.*, 2011). Thus, we measured CK levels in WT, *tps22* and transgenic plants in response to Se stress. As shown in Fig. 6A, CK content in *tps22* plants was lower than that in the WT, COM1 and OE1 under Se stress. Accordingly, a significantly lower transcript level of isopentenyl transferase (IPT), which

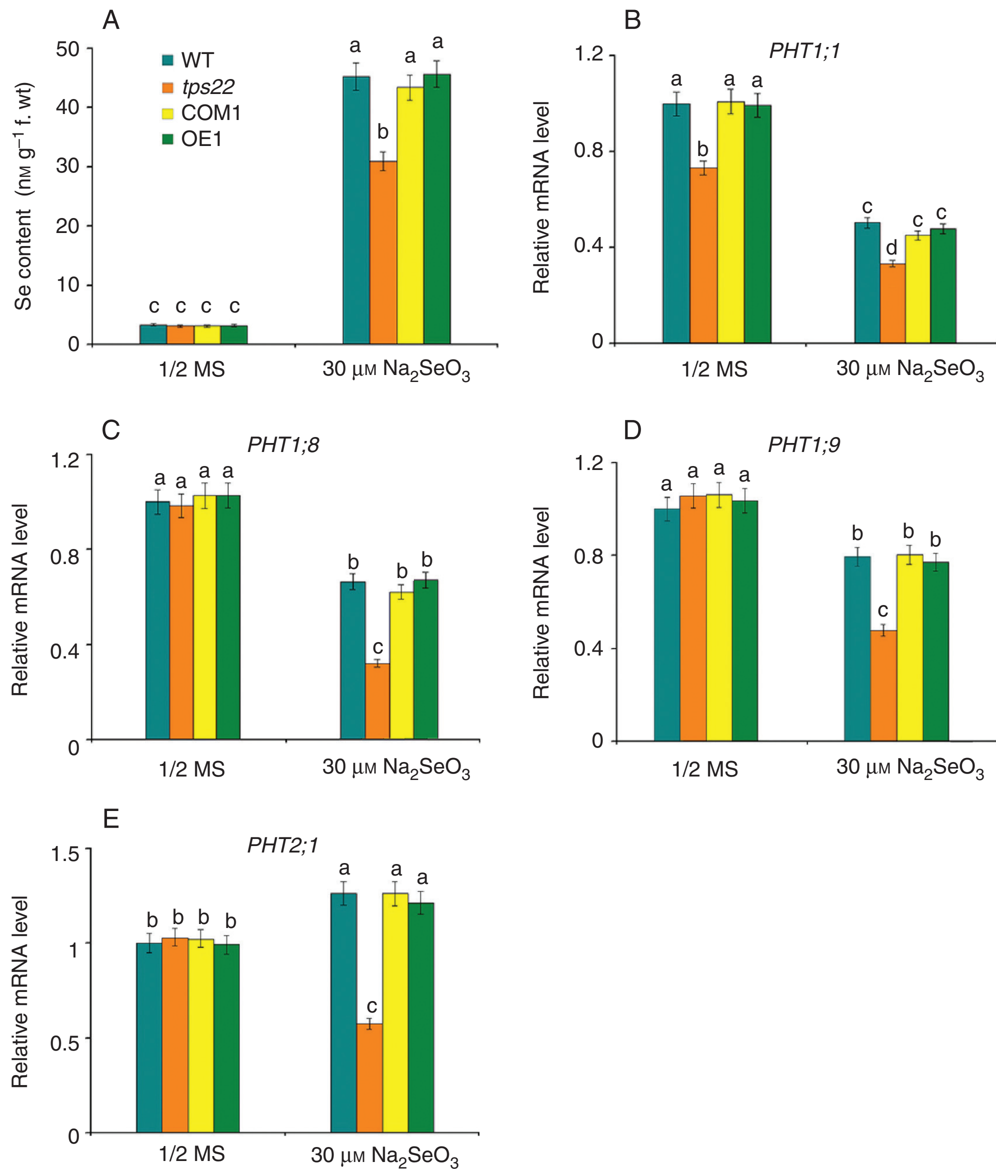

FIG. 3. Loss-of-function of *TPS22* resulted in reduced Se accumulation. (A) Se contents in WT, *tps22*, COM1and OE1 plants grown on ½ MS media with or without 30 µM Na_2SeO_3 for 2 weeks. (B–E) qRT-PCR analysis of genes involved in Se uptake, including *PHT1;1* (B), *PHT1;8* (C), *PHT1;9* (D) and *PHT2;1* (E). WT, *tps22* and transgenic lines were grown vertically on ½ MS media with or without 30 µM Na_2SeO_3 for 2 weeks, and then their mRNAs were isolated for qRT-PCR analysis. *ACTIN11* was used as the internal control. Data are the mean ± s.e. of three independent replicates. Significant differences ($P < 0.05$) between parameters are indicated by different lowercase letters.

catalyses the rate-limiting step of CK biosynthesis, was detected in mutant seedlings than in WT and transgenic plants (Fig. 6B). In Arabidopsis, CK signalling involves a multistep two-component signalling system comprising CK receptor histidine kinases (AHK2, AHK3 and AHK4), histidine phosphotransfer proteins (AHP1–5) and response regulators (type A and type B ARRs) (Kieber and Schaller, 2014). We found that even without Se treatment, transcript levels of *AHK3* and *AHK4* were lower in *tps22* than those in the WT. Addition of 30 µM Na_2SeO_3 to the medium reduced *AHK3* and *AHK4* transcription in the *tps22*, WT and transgenic seedlings, and increased *AHK2* transcription in WT and transgenic plants but not in *tps22* plants (Fig. 6C–E). The type-A ARRs are primary response genes for the CK signalling pathway because their expression is rapidly induced as a negative feedback loop (Muller and Sheen, 2007; Kieber and Schaller, 2014). As expected, we detected significantly higher *ARR3*, *ARR15* and *ARR16* transcript levels in the *tps22* than in WT and transgenic plants in response to Se stress (Fig. 6F–H). These results suggest that CK, acting through the two-component signalling pathway, might be involved in the regulation of *TPS22*-mediated Se tolerance.

Next, we tested whether application of exogenous CK had any effects on *TPS22*-mediated Se tolerance. WT and *tps22* seedlings grown on ½ MS media with or without BA were challenged by Se stress. In BA-containing medium, root growth

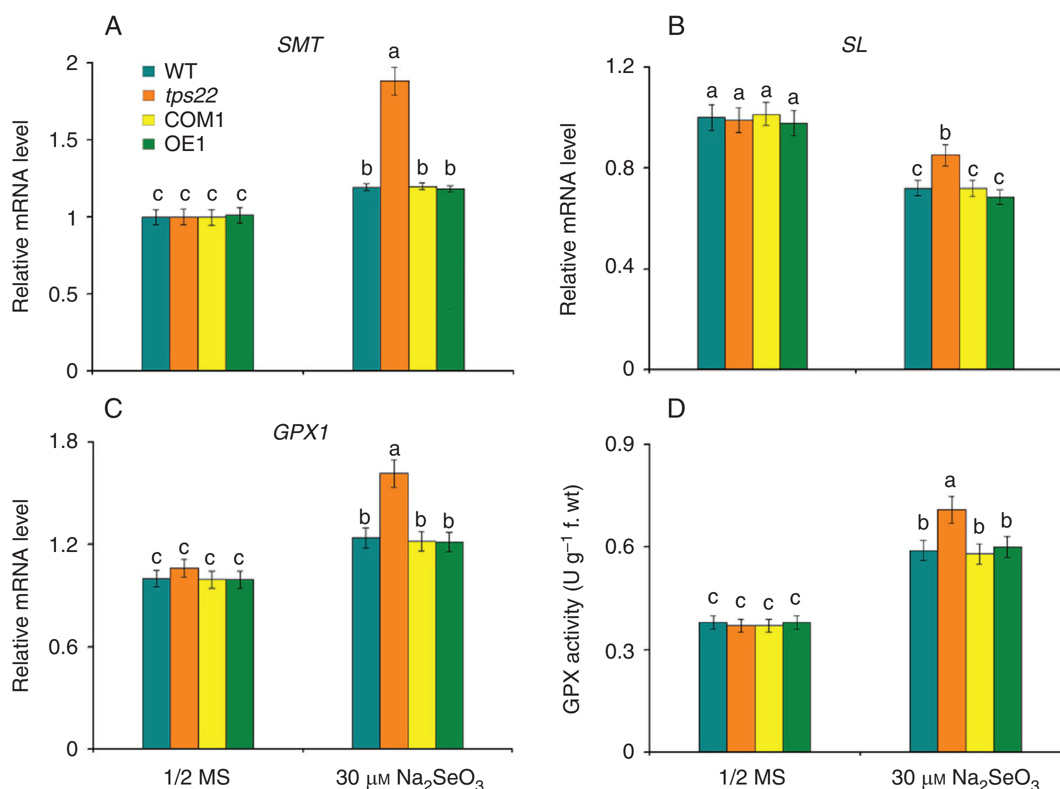

FIG. 4. Loss-of-function of *TPS22* increased Se metabolism detoxification ability. (A–C) qRT-PCR analysis of genes involved in Se metabolism detoxification, including *SMT* (A), *SL* (B) and *GPX1* (C). WT, *tps22* and transgenic lines were grown vertically on ½ MS media with or without 30 μM Na_2SeO_3 for 2 weeks, and then their mRNAs were isolated for qRT-PCR analysis. *ACTIN11* was used as the internal control. (D) GPX activity of WT, *tps22*, COM1 and OE1 plants subjected to Se for 2 weeks. Data are the mean ± s.e. of three independent replicates. Significant differences ($P < 0.05$) between parameters are indicated by different lowercase letters.

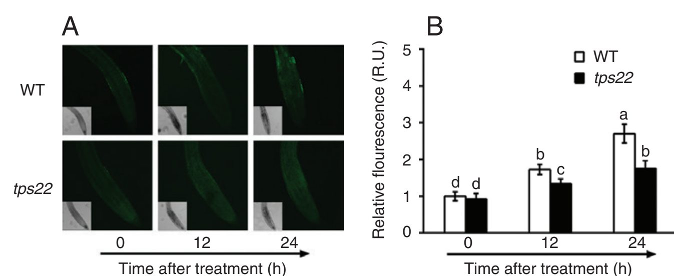

FIG. 5. ROS production induced by Se. (A) ROS production in seedling root tips of the WT and *tps22* mutant with 30 μM Na_2SeO_3 treatment for 12 and 24 h. Scale bar = 50 μm. (B) Relative fluorescence units (R.U.) of ROS in root tips. WT and *tps22* plants were grown vertically on ½ MS media for 7 d and treated with 30 μM Na_2SeO_3 for 12 and 24 h. The roots were immediately infiltrated with 20 μM H_2DCFDA fluorescent probe, and ROS were detected by laser scanning confocal microscopy. Data are the mean ± s.e. of three independent replicates. Significant differences ($P < 0.05$) are indicated by different lowercase letters.

was inhibited more severely in *tps22* than in WT seedlings, and upon Se challenge, the root length of *tps22* plants was less reduced than that of WT plants. When BA was added together with Se, root growth was inhibited severely in both WT and *tps22* seedlings and Se tolerance was abolished in *tps22* seedlings (Fig. 7). In addition, we also measured the growth of *ipt1 3 5 7* mutants (Miyawaki *et al.*, 2006) under Se stress. The *ipt1 3 5 7* plants, carrying a quadruple mutation in the key enzyme of CK biosynthesis, had longer roots than the WT on ½ MS media, and showed greater resistance than WT in the presence of Na_2SeO_3 (Fig. S4). These results suggest that CK plays a negative role in the response to Se stress.

To understand the mechanism underlying CK involved in the regulation of Se tolerance, we determined the effects of BA on Se accumulation under Se stress. BA application significantly enhanced Se accumulation in both WT and *tps22* plants and no obvious differences between the WT and *tps22* plants grown on ½ MS media containing Na_2SeO_3 (Fig. 8A). We also examined the effects of BA on the key factors of Se uptake and metabolism detoxification. As shown in Fig. 8B–D, BA significantly enhanced *PHT1;1*, *PHT2;1* and *SMT* transcription in *tps22* seedlings grown on ½ MS media with or without Na_2SeO_3, suggesting that enhanced Se accumulation in *tps22* plants was associated with activation of the transporters, especially *PHT2;1*. In addition, endogenous CK content and *IPT1* transcription were correspondingly increased in both WT and *tps22* plants with BA treatment (Fig. 8E, F). TPS25 is the related protein of TPS22 and both enzymes are sesquiterpene synthases that appear to be localized to mitochondria (Tholl and Lee, 2011). *TPS25* transcription increased more in *tps22* than in WT plants under BA treatment (Fig. S5), which might affect endogenous CK level (Fig. 8E) and result in *tps22* seedlings being more sensitive to BA than the WT (Fig. 7).

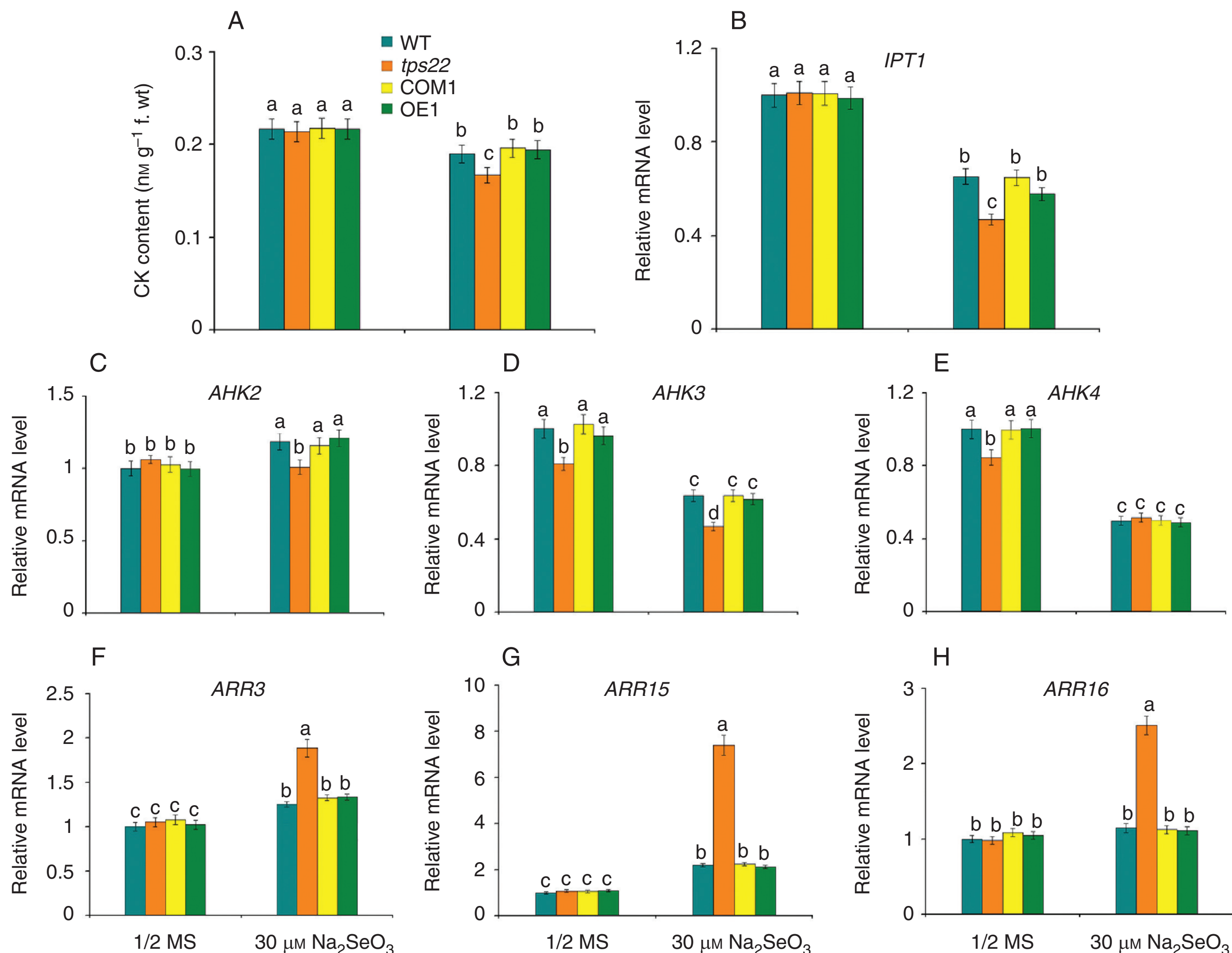

Fig. 6. Cytokinin (CK) is involved in *TPS22*-mediated Se tolerance. (A) CK contents in WT, *tps22*, COM1 and OE1 plants grown on ½ MS media with or without 30 μM Na_2SeO_3 for 2 weeks. (B–H) qRT-PCR analysis of genes involved in CK biosynthesis and CK signalling, including *IPT1* (B), *AHK2* (C), *AHK3* (D), *AHK4* (E), *ARR3*(F), *ARR15*(G) and *ARR16* (H). WT, *tps22* and transgenic lines were grown vertically on ½ MS media with or without 30 μM Na_2SeO_3 for 2 weeks, and then their mRNAs were isolated for qRT-PCR analysis. *ACTIN11* was used as the internal control. Data are the mean ± s.e. of three independent replicates. Significant differences ($P < 0.05$) between parameters are indicated by different lowercase letters.

Together, the above results suggest that CK, through affecting the key factors of Se uptake and metabolism detoxification, regulates *TPS22*-mediated Se tolerance.

GSH is required for TPS22-mediated Se tolerance

Reduction of selenite in plants might be mediated by glutathione (Van Hoewyk, 2013). To test whether the *TPS22*-mediated Se resistance was related to GSH, we compared the growth of the *tps22* mutant and WT plants in media containing BSO, an inhibitor of GSH synthesis. In ½ MS or 0.1 mM BSO-containing medium, growth of *tps22* and WT plants was similar. In the 30 μM-Na_2SeO_3-containing medium, *tps22* plants showed higher root length and fresh weight compared to the WT, but when BSO was added the growth of *tps22* plants was similar to that of the WT and the tolerance to Na_2SeO_3 disappeared (Fig. 9A–C). Moreover, the *tps22* seeedlings showed a higher increased GSH content and a higher transcript level of *GSH1*, the key factor controlling GSH synthesis (Noctor *et al.*, 2012), relative to WT plants (Fig. 9D, E).These results indicate that GSH is required for *TPS22*-mediated Se tolerance.

DISCUSSION

The Arabidopsis TPS superfamily of 40 terpenoid synthase genes consists of seven different subfamilies. AtTPS22 protein is in the TPS-a family (Aubourg *et al.*, 2002; Tholl and Lee, 2011). In this study, we explored the role of *TPS22* in Se tolerance in Arabidopsis based on genetic evidence of loss-of-function of *TPS22* plant responses to Se stress. Our results demonstrated that *TPS22* mediated Se tolerance through the CK signalling pathway. Se stress downregulated *TPS22*, which then reduced endogenous CK level. Reduced CK, acting through the two-component signalling pathway, affected the key factors of Se uptake and metabolism detoxification, and led to reduction

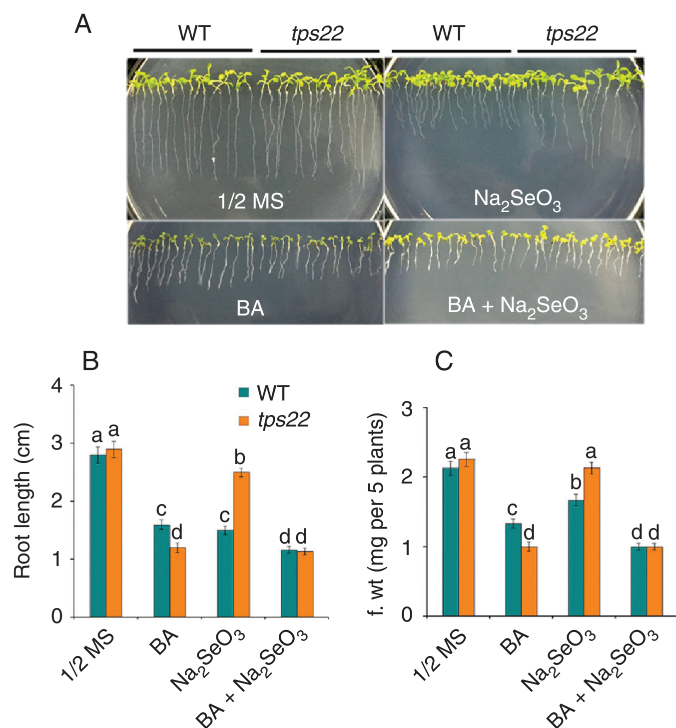

FIG. 7. Cytokinin (BA) treatment reduced Se tolerance. (A) Analysis of Se tolerance in WT and *tps22* seedlings in the absence or presence of Se and BA. WT and *tps22* lines were grown on ½ MS media with or without 30 µM Na_2SeO_3 in the absence or presence of 0.01 µM BA for 12 d. (B,C) Root length (B) and fresh weight (C) of the plants described in A. Data are the mean ± s.e. of three independent replicates. Significant differences ($P < 0.05$) are indicated by different lowercase letters.

of Se uptake and activation of metabolism detoxification. In addition, increased GSH alleviated the toxicity of excess Se. This cascade of events resulted in reduced Se accumulation and enhanced Se tolerance (Fig. 10).

Se is an essential micronutrient for many life forms yet is toxic to organisms at high tissue levels (Pilon-Smits *et al.*, 2017). In plants, selenite uptake and Se accumulation were correlated with phosphate transporters (Li *et al.*, 2008; Zhang *et al.*, 2014; Schiavon and Pilon-Smits, 2017). Here, the lower expression level of Pi transporters contributed to decreased Se content and enhanced Se resistance in *tps22* mutants under Se stress (Fig. 3). Relative to other micronutrients, the window between Se adequacy and Se toxicity is very narrow. Se toxicity, for plants, is both due to oxidative stress and because Se-amino acids are non-specifically incorporated into proteins, resulting in protein inactivation or denaturation (Pilon-Smits *et al.*, 2017). Therefore, plants have evolved different strategies to cope with Se toxicity. One of the most important mechanisms is the conversion of Se-amino acids into less harmful volatile compounds. SMT has been proposed to play a crucial role in Se detoxification given its high efficiency in methylation of selenocysteine to convert Se to less toxic forms (Lyi *et al.*, 2005; Cakir and Ari, 2013). Higher expression of the *SMT* gene led to a significant increase in Se tolerance, accumulation and volatilization in both Arabidopsis and Indian mustard (LeDuc *et al.*, 2004, 2006). BoSMT (Broccoli SMT) expression is upregulated by selenate treatment (Lyi *et al.*, 2005). In this study, we detected significantly higher transcription levels of *SMT* in *tps22* plants in response to Se stress (Fig. 4), which contributed to enhanced Se resistance in the *tps22* mutant.

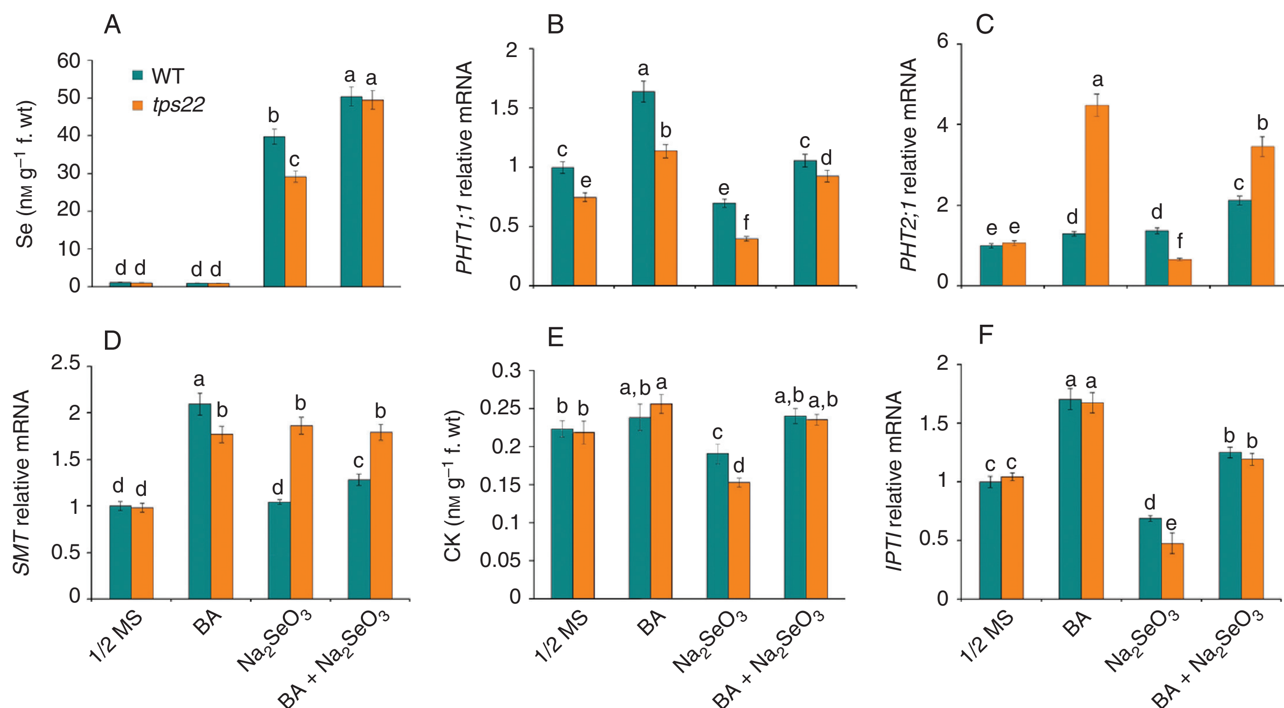

FIG. 8. Effects of exogenous cytokinin (BA) on Se uptake and assimilation. (A) Se contents in WT and *tps22* seedlings in the absence or presence of Se and BA. (B–D) qRT-PCR analysis of genes involved in Se uptake and assimilation, including *PHT1;1* (B), *PHT2;1* (C) and *SMT* (D). (E) CK contents in WT and *tps22* seedlings in the absence or presence of Se and BA. (F) qRT-PCR analysis of the *IPT1* gene. WT and *tps22* lines were grown on ½ MS media with or without 30 µM Na_2SeO_3 in the absence or presence of 0.01 µM BA for 12 d, and seedlings were sampled to analyse the index. Data are the mean ± s.e. of three independent replicates. Significant differences ($P < 0.05$) between parameters are indicated by different lowercase letters.

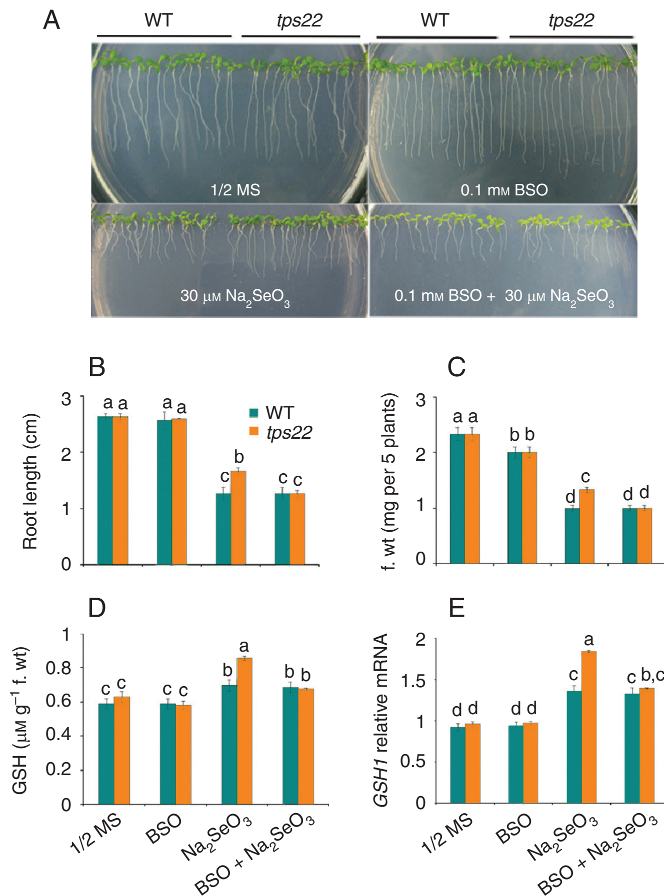

FIG. 9. GSH is required for *TPS22*-mediated Se tolerance. (A) Effects of buthionine sulfoximine (BSO) on the growth of WT and *tps22* plants. WT and *tps22* lines were grown on ½ MS media in the absence or presence of 0.1 mM BSO or 30 μM Na_2SeO_3 for 10 d. (B,C) Root length (B) and fresh weight (C) of the plants described in A. (D,E) GSH contents (D) and *GSH1* transcripts (E) in WT and *tps22* seedlings in the absence or presence of 0.1 mM BSO or 30 μM Na_2SeO_3 for 10 d. Data are the mean ± s.e. of three independent replicates. Significant differences ($P < 0.05$) are indicated by different lowercase letters.

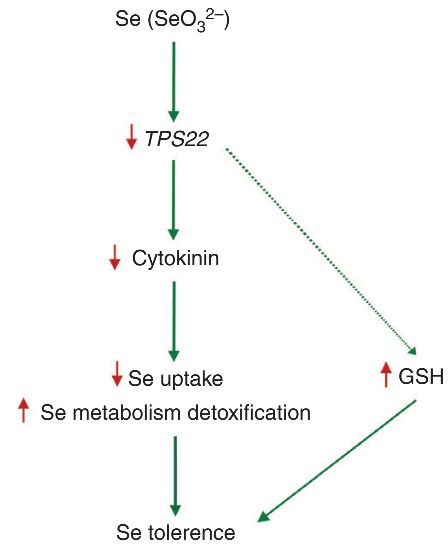

FIG. 10. A working model for *TPS22*-mediated Se tolerance. Se stress down-regulated *TPS22* expression, which reduced endogenous cytokinin level. Reduced cytokinin, acting through the two-component signalling pathway, decreased Se uptake and increased Se metabolism detoxification ability by affecting expression of the key genes. In addition, increased GSH alleviated the toxicity of excess Se. These events resulted in reduced Se accumulation and enhanced Se tolerance. Downward red arrows indicate decrease, while upward red arrows indicate increase.

Phytohormones play an important role in Se resistance. Ethylene, jasmonic acid and salicylic acid signal act to induce Se uptake and Se metabolic and stress-related genes (Freeman *et al.*, 2010; Tamaoki *et al.*, 2008*a*; Pilon-Smits *et al.*, 2017). Recent research has shown that CK is involved in the regulation of abiotic stress responses through a complex network of CK signalling (Argueso *et al.*, 2009; Ha *et al.*, 2012; Nishiyama *et al.*, 2012; Jeon & Kim, 2013; Zwack *et al.*, 2013; Li *et al.*, 2016). It is generally considered that CKs play a negative role in plant adaptation to stress by a reduction of active CK levels or by repression of CK signalling components (Ha *et al.*, 2012; Kieber and Schaller, 2014; Zwack and Rashotte, 2015). Thus, the reduced level of CK in the *tps22* mutant led to increased root length and tolerance compared with the WT subjected to Se. The lower *AHKs* and significantly higher *ARR3*, *ARR15* and *ARR16* transcript levels in the *tps22* mutant suggest that enhanced Se tolerance in *tps22* is due to repression of *AHKs* and negative feedback of the type-A ARR3, ARR15 and ARR16 (Figs 1 and 6). CK treatment increased sodium accumulation in Arabidopsis seedlings (Mason *et al.*, 2010). The expression of the high-affinity potassium transporter AtHKT1.1, which removes sodium from the root, was repressed by CK treatment (Mason *et al.*, 2010). In contrast, our results showed that transcriptions of phosphate transporters PHT1;1 and PHT2;1 were upregulated by CK treatment (Fig. 8), which increased Se accumulation, and as a result, the resistance of the *tps22* mutant to Se was completely altered and disappeared.

Selenite treatment induces oxidative stress. GSH is associated with improved tolerance to oxidative stress and Se stress in plants (Noctor *et al.*, 2012; Jiang *et al.*, 2016). GSH-mediated reduction of selenite, the reduction of selenite to selenide, may be an interaction between selenite and reduced GSH (Schiavon and Pilon-Smits, 2017). Our BSO test indicated that *TPS22*-mediated Se tolerance also occurs through the GSH detoxification pathway, by which the expression level of *GSH1* was improved and the content of GSH was increased to alleviate Se toxicity. In addition, *GPX1* transcript and GPX activity in *tps22* plants increased more to cope with ROS compared to those in the WT, and therefore we observed lower ROS accumulation in the mutant under Se stress (Figs 4 and 5).

In summary, our results suggest that loss-of-function of *TPS22* resulted in decreased Se accumulation and enhanced Se tolerance in Arabidopsis. CK is involved in *TPS22*-mediated Se tolerance by affecting expression of the key genes of Se uptake and Se metabolism detoxification.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Table S1: Primer sequences used for RT-PCR and qRT-PCR. Fig. S1: Growth of wild-type (WT) and *tps22* plants in soil-filled pots. Fig. S2:

Phenotypes of WT, *tps22* and their F_1 plants grown on ½ MS media in the absence or presence of 30 µM Na_2SeO_3 for 3 weeks. Fig. S3: Pi contents in WT and *tps22* plants grown on ½ MS media with or without 30 µM Na_2SeO_3 for 2 weeks. Fig. S4: The phenotypes of WT and *ipt1 3 5 7* plants grown on ½ MS media with or without 30 µM Na_2SeO_3 for 2 weeks. Fig. S5: *TPS25* transcript level determined by qRT-PCR analysis.

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