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APS and APR gene families in *Broussonetia papyrifera*: evolutionary conservation, functional divergence, and roles in selenium assimilation and biofortification

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Abstract

Selenium (Se) biofortification in forage tree species offers a sustainable strategy to improve nutritional quality. This study investigated the physiological and molecular responses of *Broussonetia papyrifera*, a high-biomass woody species, to exogenous selenite (Na_2SeO_3) and selenate (Na_2SeO_4). Low Se concentrations (≤ 0.4 mM) significantly enhanced plant growth, while higher concentrations (0.8 mM), especially Na_2SeO_4 , inhibited biomass accumulation in a dose-dependent manner. Both forms of Se substantially increased foliar Se content, with Na_2SeO_4 showing higher uptake efficiency. Genome-wide screening identified four sulfate/ Na_2SeO_4 assimilation-related genes including ATP sulfurylase 1 (*BpAPS1*), *BpAPS2*, adenosine 5'-phosphosulfate reductase (*BpAPR1*), and *BpAPR2*. Phylogenetic analyses confirmed the evolutionary conservation of these proteins, which were localized to the chloroplasts. Tissue-specific expression patterns revealed a positive correlation between transcript levels of *BpAPS1*, *BpAPR1*, and *BpAPR2* and Se content, while *BpAPS2* expression was inversely correlated, indicating functional divergence. Compared to wild-type (WT) plants, transgenic *Arabidopsis thaliana* lines overexpressing these genes accumulated 1.27- to 1.60-fold more Se under Na_2SeO_4 stress, accompanied by coordinated upregulation of key endogenous Se metabolism genes like *AtAPS*, *AtAPR*, *S-adenosylmethionine carriers 3* (*AtSAM*), *homocysteine S-methyltransferase* (*AtHMT*), *methylselenol methyltransferase* (*AtMMT*), suggesting enhanced Se assimilation and detoxification capacity. Collectively, these findings clarify the functional roles of the APS and APR gene families in Se uptake, assimilation, and detoxification in a woody crop, highlighting potential genetic targets for Se biofortification.

Keywords *Broussonetia papyrifera*, ATP sulfurylase, Adenosine 5'-phosphate reductase, Selenium biofortification

Introduction

Selenium (Se) is an essential trace element for humans and animals, playing critical roles in antioxidant defense, thyroid hormone metabolism, and immune regulation (Winther et al. 2020). Since Se cannot be synthesized endogenously by humans and other animals, it must be obtained through dietary sources (Wang et al. 2021). However, Se concentrations in most natural foods are generally low (Abdullah et al. 2025). Plants, as the primary link in the soil–plant–human food chain, absorb inorganic Se from the soil and convert it into bioavailable

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organic forms for higher trophic levels (Huang et al. 2023; Liao et al. 2024). In China, widespread Se deficiency in soils results in low Se content in feed crops, limiting Se nutrition in livestock and poultry (Duborská et al. 2022).

The Se bioavailability of plants is largely determined by their ability to assimilate inorganic Se into organic compounds *via* biochemical pathways associated with sulfur metabolism (Yarmolinsky et al. 2013). Key enzymes in this process include ATP sulfurylase (APS) and adenosine 5'-phosphosulfate reductase (APR), which catalyze the activation of sulfate/ Na_2SeO_4 to adenosine 5'-phosphosulfate and its subsequent reduction, providing reduced sulfur/Se for incorporation into selenoamino acids (Pilon-Smits et al. 1999). In *Brassica oleracea*, exposure to Na_2SeO_4 upregulates APS and APR activities, enhancing Se uptake and conversion, thereby increasing Se accumulation and nutritional value (Gui et al. 2022). Transcriptomic studies have demonstrated that APS and APR gene expression in *Broussonetia papyrifera* is induced by exogenous Se in a concentration-dependent manner, suggesting their involvement in Se assimilation (Zhu et al. 2025). Despite extensive research on Se biofortification in staple food crops, forage species such as *B. papyrifera* remain underexplored, though they hold significant potential. The regulatory mechanisms and functional characteristics of APS and APR family members in this species, however, remain largely unknown.

B. papyrifera, a fast-growing woody forage species, exhibits favorable agronomic traits, including high biomass yield, strong ecological adaptability, and remarkable tolerance to various abiotic stresses, including Se stress (Li et al. 2025; Chen et al. 2025). Notably, *B. papyrifera* demonstrates a significant ability to absorb inorganic Se and convert it into organic forms, a key factor contributing to its Se stress tolerance, making it a promising candidate for Se-biofortified forage development (Chen et al. 2024). The leaves of *B. papyrifera*, rich in protein, essential amino acids, and minerals like Se, have long been utilized as forage and in traditional medicine, offering substantial ecological and economic value (Chen et al. 2022). Increasing organic Se content in this species presents a practical, safe, and cost-effective strategy to mitigate Se deficiency in animal production systems (Yuan et al. 2024). However, a limited understanding of the molecular mechanisms regulating Se uptake, transport, assimilation, and transformation in *B. papyrifera*, particularly the conserved yet underexplored roles of APS and APR genes in Se metabolism, limits its broader application in Se biofortification.

In this study, four Se assimilation-related genes (*BpAPS1*, *BpAPS2*, *BpAPR1*, and *BpAPR2*) were cloned from *B. papyrifera* and characterized through structural, promoter, phylogenetic, and subcellular localization

analyses. The physiological responses of *B. papyrifera* to varying exogenous Se concentrations were examined, along with the tissue-specific expression patterns of these genes in relation to Se accumulation. Functional validation through heterologous expression in *Arabidopsis thaliana* confirmed the roles of these genes in enhancing Se uptake, tolerance, and metabolic regulation. These findings highlight the functional significance of the APS and APR gene families in Se metabolism in *B. papyrifera*, providing valuable molecular targets for the development of Se-enriched woody forage species.

Materials and methods

Plant materials

B. papyrifera 'Kegou 101', developed by the Institute of Botany, Chinese Academy of Sciences, was obtained commercially and cultivated in a glasshouse on the western campus of Yangtze University, Jingzhou, China (30.35°N, 112.15°E). The growth substrate consisted of peat, red soil, vermiculite, and perlite mixed in a volume ratio of 6:1:1:1.

Seedlings were transplanted into horticultural pots and irrigated weekly with autoclaved Hoagland solution. After one month of acclimation, homogeneous plants were treated with Na_2SeO_4 or Na_2SeO_3 at concentrations of 0, 0.2, 0.4, or 0.8 mM (Chen et al. 2025). Each pot received 100 mL of Se solution *via* weekly foliar sprays, a volume commonly used in foliar Se application studies to ensure uniform leaf coverage without runoff, for four weeks. Roots, stems, and leaves were harvested 7 d after the final application and stored at -80°C . Three biological replicates, each with four plants, were employed.

A. thaliana seeds were germinated under a 16 h light/8 h dark cycle at 25°C and 65% relative humidity, and 15-d-old plants were treated with 0.4 mM Na_2SeO_4 *via* foliar spray for two weekly cycles. The plants were harvested 5 d after the final application and thoroughly rinsed. *Nicotiana benthamiana* seeds were germinated at 25°C , and seedlings with two true leaves were transplanted and grown under the same conditions until four to six leaves developed, after which they were used for subcellular localization assays.

Genome-wide identification and physicochemical characterization of *BpAPS* and *BpAPR* genes

Genome-wide identification of *BpAPS* and *BpAPR* genes was conducted using previously assembled *B. papyrifera* genome data. Hidden Markov models (HMMs) for the APS (PF01747) and APR (PF01507.22) domains were retrieved from Pfam (<https://pfam.xfam.org/>) and used to query the *B. papyrifera* protein database with HMMER 3.0 (Chen et al. 2024). Candidate sequences were validated for conserved domains using NCBI-CDD and

SMART, and their chromosomal locations were analyzed with TBtools-II v2.388. The physicochemical properties of the proteins were analyzed using ProtParam (<http://web.expasy.org/protparam/>), transmembrane domains were predicted with TMHMM v2.0, and signal peptides were identified with SignalP-4.1. Hydrophobicity profiles were generated via ProtScale (<http://web.expasy.org/protscale/>), secondary structures were predicted using SOPMA (https://npsa.lyon.inserm.fr/cgi-bin/secpred_sopma.pl), and tertiary structures were modeled and validated through SWISS-MODEL (<http://swissmodel.org/workspace/>) (Chen et al. 2020).

Phylogenetic analysis and promoter analysis

The BpAPS and BpAPR protein sequences in *B. papyrifera* were used to search for corresponding sequences in plants from Phytozome (<https://phytozome-next.jgi.doe.gov/>), NCBI, or GigaDB (<http://gigadb.org/>). Thirty-two species were included to generate phylogenetic trees using MUSCLE v3.8.31 with default parameters (Tables S1, S2). Phylogenetic analysis was performed using the neighbor-joining method in MEGA-X, based on full-length protein sequences (Liu et al. 2024).

Based on the previously assembled genome data of *B. papyrifera*, 2,000 bp upstream sequences of the *BpAPS* and *BpAPR* genes were extracted and used to predict cis-acting regulatory elements via the PlantCARE online tool (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). The identified *cis*-elements were visualized using TBtools (Chen et al. 2020).

Subcellular localization

To analyze the subcellular localization of BpAPS1, BpAPS2, BpAPR1, and BpAPR2, green fluorescent protein (GFP) fusion constructs were generated and introduced into *Agrobacterium tumefaciens* GV3101, along with the chloroplast marker p1300-35S-OsPHT4;1-eGFP (Li et al. 2020). The *Agrobacterium* suspensions were coinfiltrated into the leaves of 4-week-old *N. benthamiana* plants for transient expression. After 48 h of incubation under a 16 h light/8 h dark photoperiod at 25°C, GFP fluorescence in the epidermal cells was observed using a Leica TCS SP8 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany).

Isolation and functional vector preparation of sulfur assimilation genes

Gene-specific primers (primer sequences are listed in Table S3) were designed to amplify full-length *BpAPS* and *BpAPR* cDNAs from total RNA of *B. papyrifera*.

First-strand cDNA was synthesized for gene cloning (RNA sample details provided in Table S4). PCR products were purified, ligated into the pMD-19 T vector, transformed into *Escherichia coli* DH5 α , and screened by colony PCR using M13 primers. Positive clones were confirmed by Sanger sequencing (Sangon Biotech, Shanghai) and alignment with transcriptome-derived sequences using SnapGene software (GSL Biotech LLC, Chicago, IL, USA). Verified coding regions were subcloned and inserted into the overexpression vector pNC-Cam2304-MCS-35S and the subcellular localization vector pNC-Cam1304-SubC via homologous recombination. The recombinant plasmids were propagated in DH5 α and introduced into *A. tumefaciens* GV3101 via the freeze–thaw method. Transformed colonies were selected on YEP medium containing kanamycin and rifampicin and validated by PCR using gene-specific primers (Guo et al. 2024).

Determination of total selenium content

Transgenic *A. thaliana* plants overexpressing *BpAPS* and *BpAPR* were generated by floral dip using GV3101 carrying pNC-Cam2304-MCS-35S constructs (Table S5). Wild-type (WT) plants and three independent homozygous T3 overexpression lines (OE1–OE3) were grown in 90-cell trays in a controlled growth chamber under a 16 h light/8 h dark photoperiod. Foliar Se treatment began approximately 7 d after bolting. Plants were sprayed with 0.4 mmol·L^{−1} Na₂SeO₄, with three applications at 5-d intervals. Plant tissues were harvested after the final treatment for Se analysis.

Approximately 0.2 g of plant material was digested with HNO₃–H₂O₂ using a microwave digestion system, followed by HCl treatment and clarification on a graphite digestion block. After cooling, potassium ferri-cyanide was added, and the samples were diluted to volume with deionized water. Total Se concentrations were determined by hydride generation–atomic fluorescence spectrometry (HG–AFS), using 5% (v/v) HCl as the carrier solution and KBH₄/KOH as the reducing agent, as previously described (Rao et al. 2021).

Gene expression analysis of selenium-associated genes in transgenic *A. thaliana*

To assess the expression of Se-related genes in transgenic *A. thaliana*, genome and annotation data (TAIR10) were obtained from the Ensembl Plants database (https://plants.ensembl.org/Arabidopsis_thaliana/Info/Index). Genes encoding key enzymes in Se metabolic pathways were screened, with candidates selected based on an E-value threshold of < 1e−5. Corresponding HMMs were used to extract gene sequences from the genome

files. Total RNA was isolated from transgenic *A. thaliana* leaves, and cDNA was synthesized for quantitative real-time PCR (qRT-PCR) analysis. Expression levels of the candidate genes were quantified using SYBR Green chemistry, with *AtActin* as the internal reference gene, and relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

Statistical analysis

Statistical analysis was performed with Excel (v2022) or Origin (<https://www.originlab.com/>), and results are presented as the mean $\pm$ SE of three biological replicates. One-way ANOVA followed by Tukey's test was applied for intergroup significance, with $P < 0.05$ considered statistically significant.

Results

Characterization and phylogenetic analysis of *BpAPS* and *BpAPR* genes

Using HMMs of the APS (PF01747) and APR (PF01507.22) domains from the Pfam database, two candidate APS and two APR protein sequences were identified by searching the *B. papyrifera* protein database with HMMER 3.0. Conserved domain analysis using the NCBI CDD tool confirmed that both APS proteins contained the ATP-sulfurylase family domain, while both APR proteins harbored the 5'-adenylylsulfate reductase family domain (Fig. 1a). Chromosomal localization analysis revealed that *BpAPS1*, *BpAPS2*, *BpAPR1*, and *BpAPR2* were located on chromosomes 2, 13, 8, and 5, respectively (Fig. 1b). Based on these results, two members from each gene family were identified and designated as *BpAPS1*, *BpAPS2*, *BpAPR1*, and *BpAPR2*.

The full-length cDNA sequences of *BpAPS1*, *BpAPS2*, *BpAPR1*, and *BpAPR2* ranged from 1401 to 1446 bp, encoding proteins of 466 to 481 amino acids. The deduced proteins comprised 20 standard amino acids, with predicted molecular weights of 51.77–53.63 kDa. Homology modeling via SWISS-MODEL revealed that *BpAPR1* shared 79.82% sequence identity with the *A. thaliana* APR1 model (PDB: 5yry.1.A), while *BpAPS1*, *BpAPS2*, and *BpAPR2* exhibited 78.36% identity to the APS model from *Glycine max* (PDB: 4maf.1.A) (Table S6). Subcellular localization prediction indicated that all four proteins were localized to chloroplasts.

Phylogenetic analysis showed that the two *BpAPS* genes clustered with APS homologs from *Morus notabilis*, *Ficus carica*, and *Cannabis sativa*, while the two *BpAPR* genes grouped with APR homologs from the same species. *BpAPS1* and *BpAPS2* showed the closest relationships to *FcAPS2* from *F. carica* and *MnAPS1* from *M. notabilis*, with 100% bootstrap support. Similarly,

BpAPR1 and *BpAPR2* exhibited the highest homology with *FcAPR1* (GMN36344.1:1–472) and *FcAPR2*, both with 100% support. *BpAPR2* was most closely related to *MnAPR2* (XP_010096894.1:22–458), with 90% support. These results suggest that *BpAPS* and *BpAPR* genes are highly conserved and likely share similar functional roles with their homologs in related species such as *M. notabilis* (Fig. 1c).

Cis-acting element analysis of the *BpAPS* and *BpAPR* promoters

Promoter analysis revealed that the upstream regions of *BpAPS1*, *BpAPS2*, *BpAPR1*, and *BpAPR2* contained *cis*-elements related to light, ABA, and MeJA responses, indicating regulation by both environmental and hormonal signals. In addition to these common elements, the *BpAPS1* promoter also included salicylic acid (SA)- and gibberellin (GA)-responsive elements, as well as binding sites for MYB and W-box transcription factors, suggesting a potential role in stress-related transcriptional regulation. The *BpAPS2* promoter uniquely contained low-temperature-responsive elements, pointing to a role in cold stress responses. Both *BpAPR1* and *BpAPR2* promoters contained GA- and MeJA-responsive elements, with the *BpAPR2* promoter also harboring defense- and stress-related *cis*-elements. In contrast, the *BpAPR1* promoter contained ABA-responsive elements but lacked SA-responsive elements (Fig. 1d).

Subcellular localization of APS and APR proteins

Confocal microscopy demonstrated that GFP fluorescence from all four fusion proteins overlapped with chlorophyll red autofluorescence and the OsPHT4;1-eGFP chloroplast marker signal, confirming the chloroplast localization of *BpAPS1*, *BpAPS2*, *BpAPR1*, and *BpAPR2* (Fig. 2). This localization suggests that these proteins may play a role in chloroplast-associated Se assimilation and regulatory pathways.

Effects of exogenous selenium treatment on *B. papyrifera* seedlings

At 0.4 mM, both Se forms continued to promote growth, although their effectiveness was lower compared to 0.2 mM. Notably, Na_2SeO_4 at this concentration caused visible leaf damage, including reddish-brown spots and mild chlorosis along the leaf edges. At lower concentrations (0.2 mM), both Na_2SeO_3 and Na_2SeO_4 promoted seedling growth, resulting in larger leaves, increased biomass, and maximum plant heights of 42.17 cm and 40.82 cm, respectively—an increase of 14.4% and 10.8% over the control. However, at high concentrations (0.8 mM) of either Se form, growth was inhibited, with

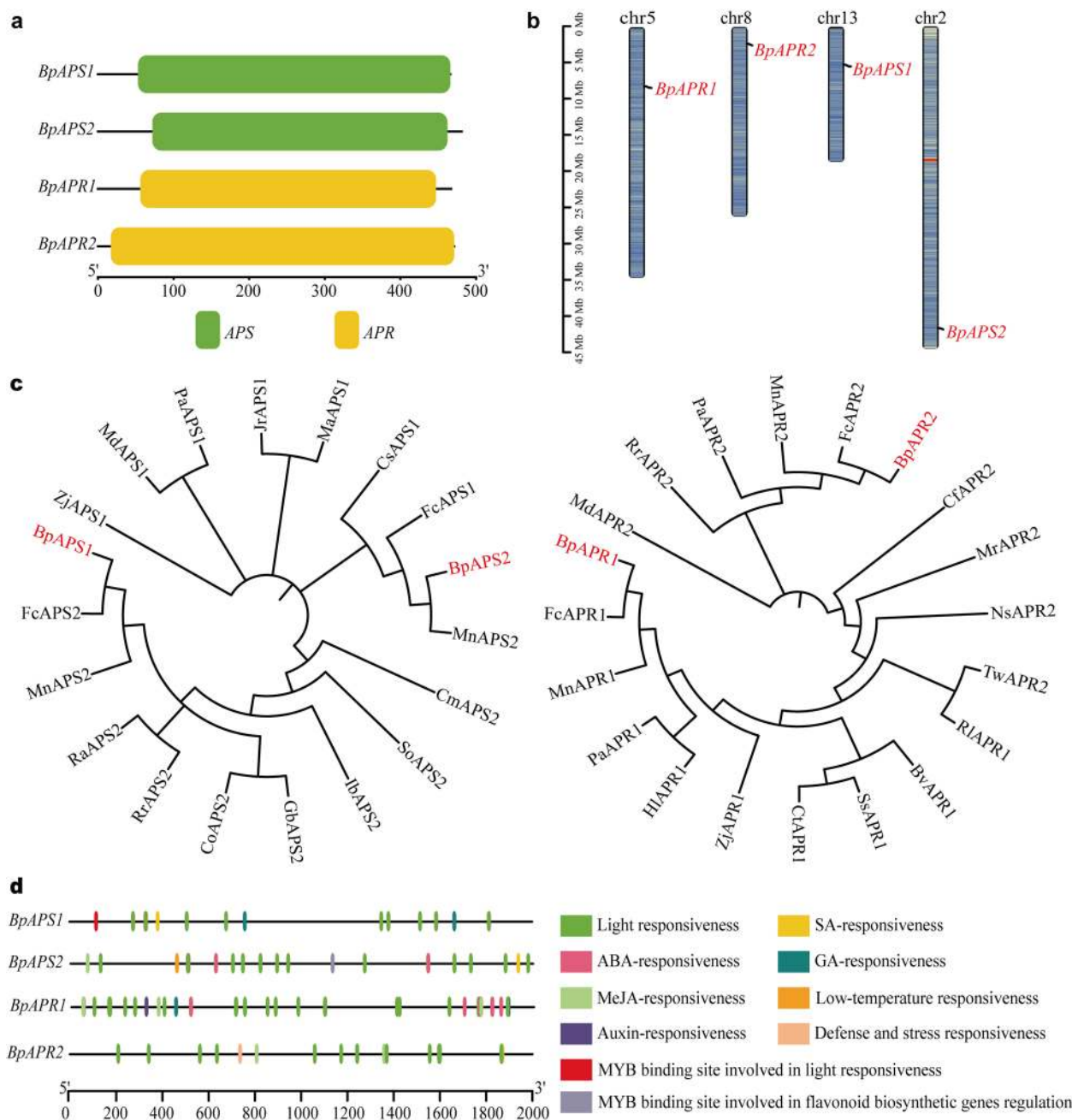


Fig. 1 Protein structure and promoter analysis of ATP sulfurylase 1 (*BpAPS*) and adenosine 5'-phosphosulfate reductase 1 (*BpAPR*) genes in *Broussonetia papyrifera*. **a** Conserved domain analysis of *BpAPS* and *BpAPR* proteins. **b** Chromosomal localization and distribution of *BpAPS1*, *BpAPS2*, *BpAPR1*, and *BpAPR2* in *B. papyrifera*. **c** Phylogenetic analysis of APS and APR proteins based on full-length amino acid sequences from various plant species. The trees were constructed using the neighbor-joining (NJ) method with 1,000 bootstrap replicates. **d** Cis-acting element analysis of the 2,000-bp upstream promoter regions of the *BpAPS* and *BpAPR* genes. ABA, abscisic acid; SA, salicylic acid; GA, gibberellin

Na_2SeO_4 causing more severe phytotoxic effects, such as leaf curling, chlorosis, and even necrosis. Compared to the control, plant height was reduced by 29.8%, suggesting a strong stress response at this dosage (Fig. 3a, b).

Expression analysis of APS and APR genes under selenium treatments

Both Na_2SeO_3 and Na_2SeO_4 treatments significantly increased the total Se concentration in all *B. papyrifera* tissues in a dose-dependent manner. Se accumulation in

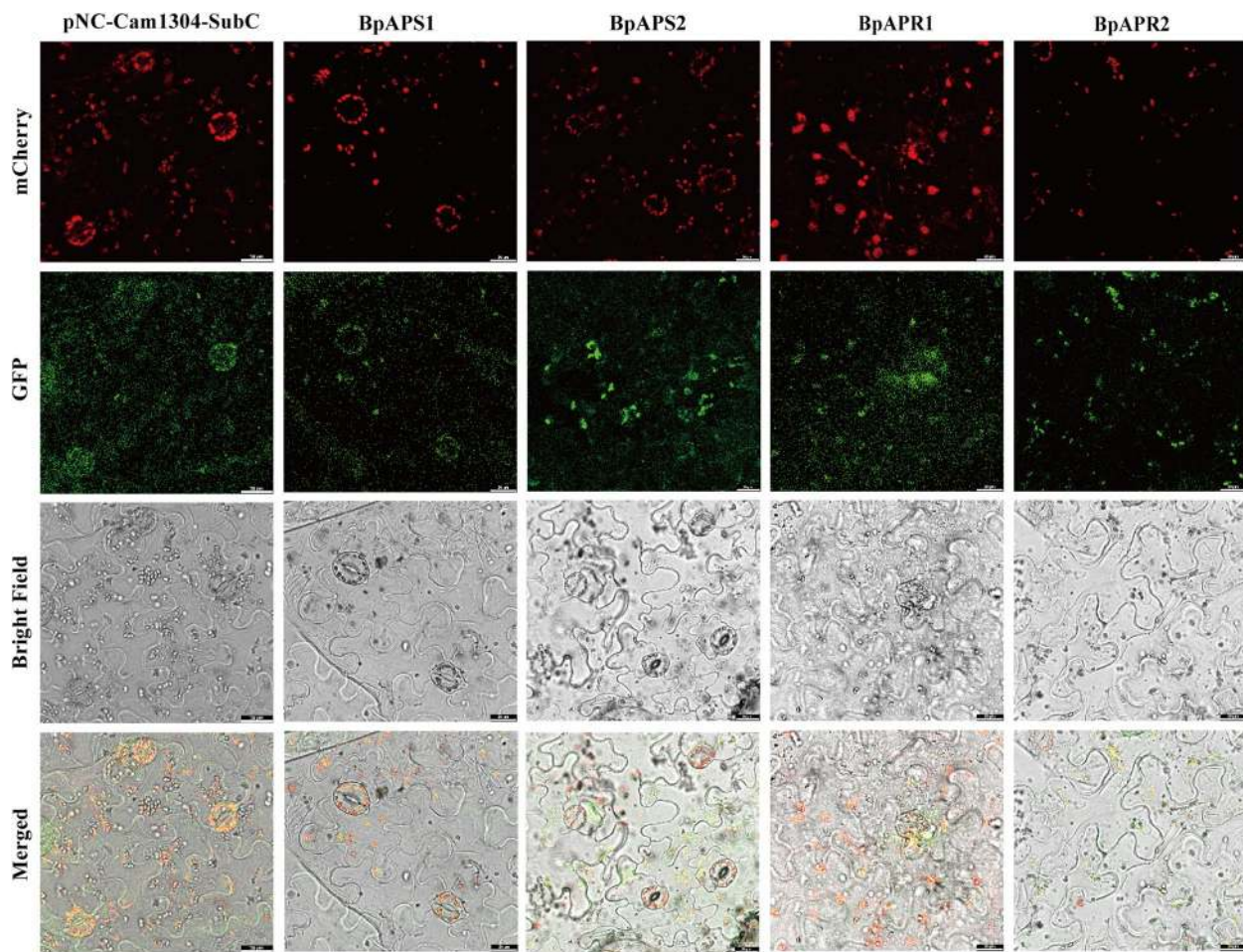


Fig. 2 Subcellular localization of ATP sulfurylase 1 (BpAPS1), BpAPS2, adenosine 5'-phosphosulfate reductase 1 (BpAPR1), and BpAPR2 from *Broussonetia papyrifera* in *Nicotiana benthamiana* leaf epidermal cells. GFP, green fluorescent protein

roots and stems plateaued under the Na_2SeO_3 treatment at 0.2–0.4 mM, whereas Se content in leaves reached its highest ($100.02 \text{ mg}\cdot\text{kg}^{-1}$ dry weight; DW) at 0.8 mM. Compared to Na_2SeO_3 , Na_2SeO_4 resulted in greater Se enrichment across tissues at equivalent concentrations, indicating enhanced uptake efficiency and tissue tolerance (Fig. 3c–e). Quantitative expression analysis revealed distinct Se-responsive patterns of *BpAPS* and *BpAPR* genes in *B. papyrifera* tissues under different Se concentrations and forms. *BpAPS1* expression peaked at low-Se concentrations (0.2 mM), while BpAPS2 was most strongly induced at 0.8 mM Se, likely reflecting elevated Se stress rather than just Se accumulation. *BpAPR* genes also exhibited tissue-specific maxima: *BpAPR1* was highest in roots at 0.2 mM and in leaves at 0.4 mM, while *BpAPR2* showed a universal peak at 0.2 mM (Fig. 4).

Overexpression of *APS* and *APR* genes elevated endogenous selenium content in transgenic plants

Following foliar application of 0.4 mM Na_2SeO_4 , all transgenic lines accumulated significantly higher levels of Se compared to WT plants (Figs. 5a, S1). The total Se content in the WT plants was $67.93 \text{ mg}\cdot\text{kg}^{-1}$ DW, while Se accumulation was markedly higher in all overexpression lines. Among these, the *BpAPS1*-OE1, *BpAPS1*-OE2, and *BpAPS1*-OE3 plants exhibited the highest Se content, averaging $108.43 \text{ mg}\cdot\text{kg}^{-1}$ DW, representing an approximately 60% increase compared to the WT. The *BpAPS2*-OE1–OE3 lines also showed strong Se enrichment, with an average Se content of $101.59 \text{ mg}\cdot\text{kg}^{-1}$ DW. In contrast, the *BpAPR1*-OE1–OE3 plants displayed a more moderate increase to $86.72 \text{ mg}\cdot\text{kg}^{-1}$ DW, approximately 1.28 times that of the

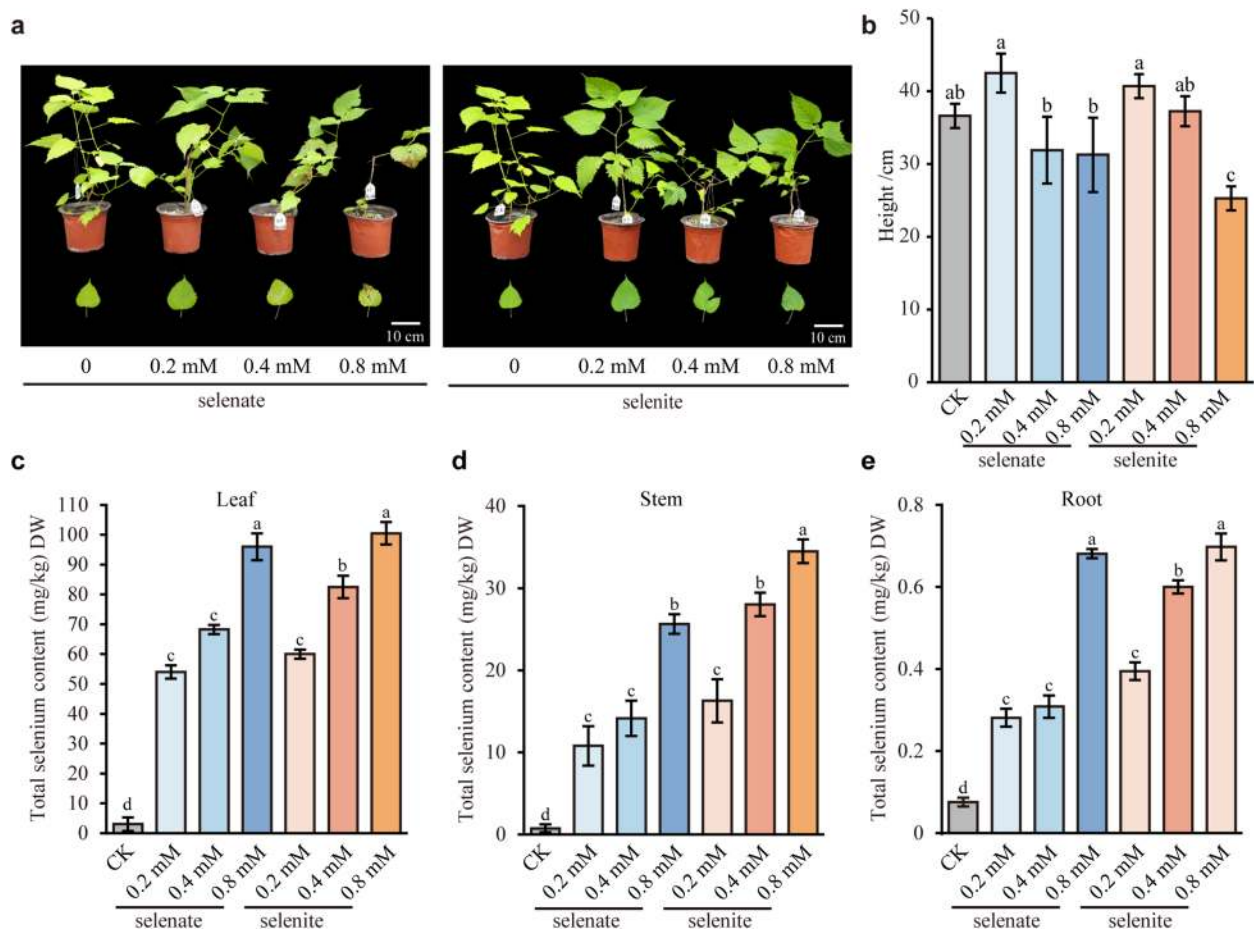


Fig. 3 Dose-dependent effects of different Selenium (Se) forms and concentrations on the growth and Se accumulation of *Broussonetia papyrifera* seedlings. **a** Growth phenotype under selenite (Na_2SeO_3) and selenate (Na_2SeO_4) treatments. **b** Plant height of *B. papyrifera* seedlings treated with various Se forms and concentrations. **c–e** Total selenium content in the leaves (**c**), stems (**d**), and roots (**e**) of *B. papyrifera* under different Se treatments. DW, dry weight. Different letters denote significant differences according to Duncan's multiple range test ($P < 0.05$)

WT. The *BpAPR2*-OE1–OE3 transgenic lines accumulated $96.34 \text{ mg} \cdot \text{kg}^{-1} \text{ DW}$ Se, approximately 1.41-fold greater than the WT. These results indicate that overexpressing these genes enhances Se accumulation in *Arabidopsis*, with *BpAPS1* having the most significant effect (Fig. 5b).

Expression profiling of genes involved in selenium metabolism in transgenic plants

Under 0.4 mM Na_2SeO_4 treatment, overexpression of *BpAPS1* and *BpAPS2* in *Arabidopsis* significantly altered the transcriptional regulation of Se assimilation pathways. The expression of multiple *AtAPS* and *AtAPR* isoforms increased in both lines, enhancing the upstream conversion of Na_2SeO_4 to activated intermediates and their subsequent reduction. In both *BpAPS1*-OE1–OE3 and *BpAPS2*-OE1–OE3 plants, genes involved in Se

activation and reduction were notably upregulated. As shown in the expression heatmaps, several *AtAPS* and *AtAPR* isoforms were coordinately induced, indicating enhanced conversion of Na_2SeO_4 into activated intermediates followed by their reduction. Specifically, *BpAPS1*-OE1–OE3 primarily promoted the expression of *AtAPS3*, *AtAPS4*, *AtAPR2*, and *AtAPR3*, whereas *BpAPS2*-OE more strongly activated *AtAPS1/2* and significantly increased *AtAPR3* expression, suggesting functional divergence between the two *APS* genes in regulating Se assimilation pathways (Fig. 6). Consistent with these transcriptional changes, genes involved in methyl-group metabolism, including *S-adenosylmethionine carriers 3 and 4* (*AtSAM3* and *AtSAM4*), were broadly upregulated in the *APS*-overexpressing lines, supporting enhanced synthesis of seleno-amino acids. Furthermore, the expression of detoxification- and volatilization-related

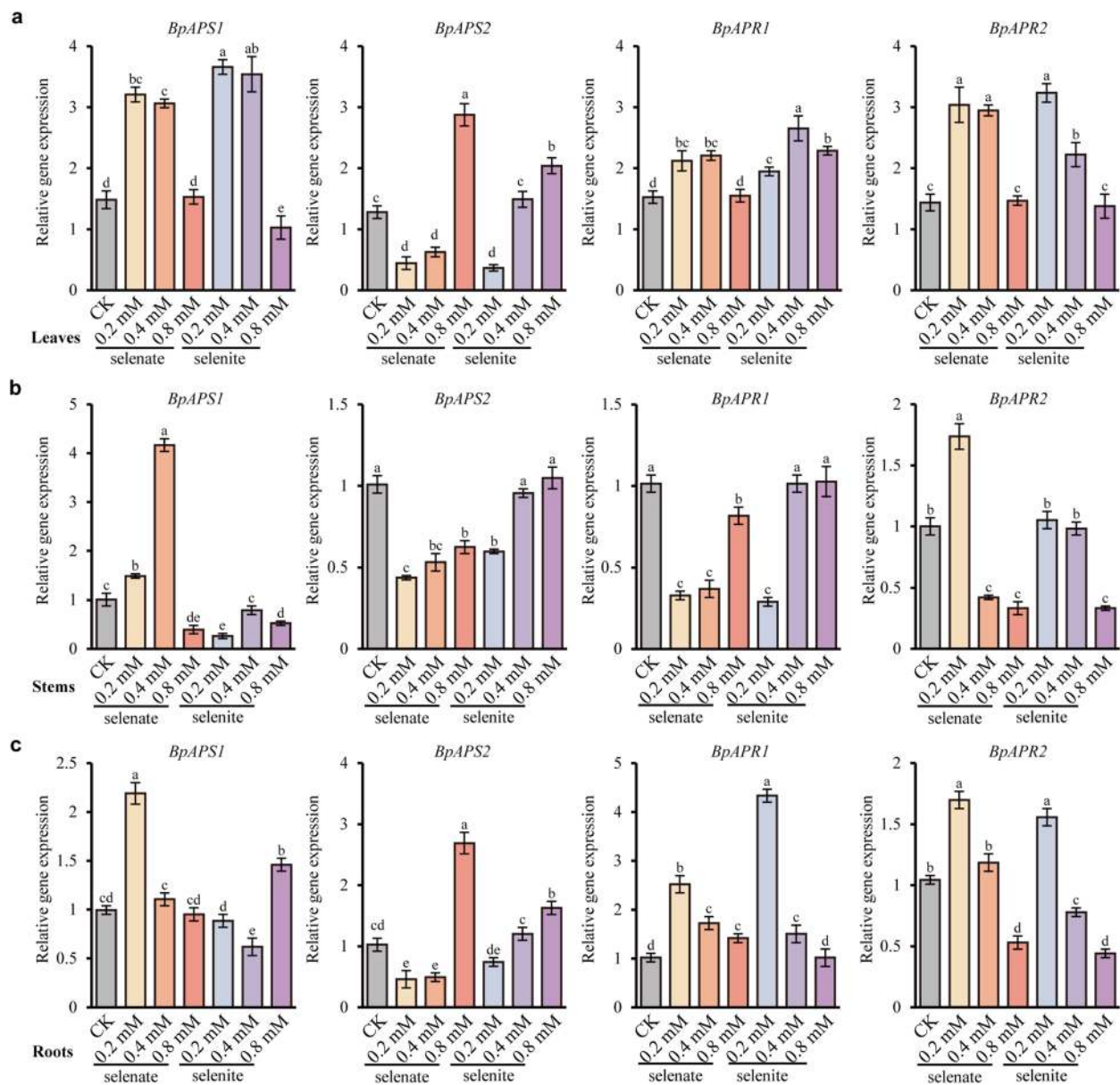


Fig. 4 Relative expression levels of ATP sulfurylase 1 (APS) and adenosine 5'-phosphosulfate reductase 1 (APR) genes in the leaves (a), stems (b), and roots (c) of *Broussonetia papyrifera* under selenium treatment. CK, control treatments. Gene expression levels were quantified by qRT-PCR and presented as relative values. Different letters represent significant differences according to Duncan's multiple range test ($P < 0.05$)

genes, such as *homocysteine S-methyltransferase 1* and 3 (*AtHMT1* and *AtHMT3*) and *methylselenol methyltransferase 1* (*AtMMT1*), was consistently induced, particularly in *BpAPS2*-OE1–OE3 plants. This suggests that accelerated Se methylation and volatilization processes may contribute to enhanced Se tolerance (Figs. 6–7).

In *BpAPR1*-OE1–OE3 and *BpAPR2*-OE1–OE3 plants, Se metabolic pathways were also reconfigured in an isoform-dependent manner. *BpAPR1*-OE1–OE3 primarily

upregulated *AtAPR2* and *AtAPR3*, reflecting an increase in reductive assimilation capacity. In contrast, *BpAPR2*-OE1–OE3 strongly activated *AtAPS1* and significantly induced *AtAPS2/4*, while repressing *AtAPR1/2* and enhancing *AtAPR3* expression, suggesting a shift toward alternative APR-mediated reduction routes. Moreover, *AtSAM1/2* was significantly upregulated in *BpAPR2*-OE1–OE3 plants, further reinforcing their Se methylation capacity, whereas *AtMMT1* was induced in both

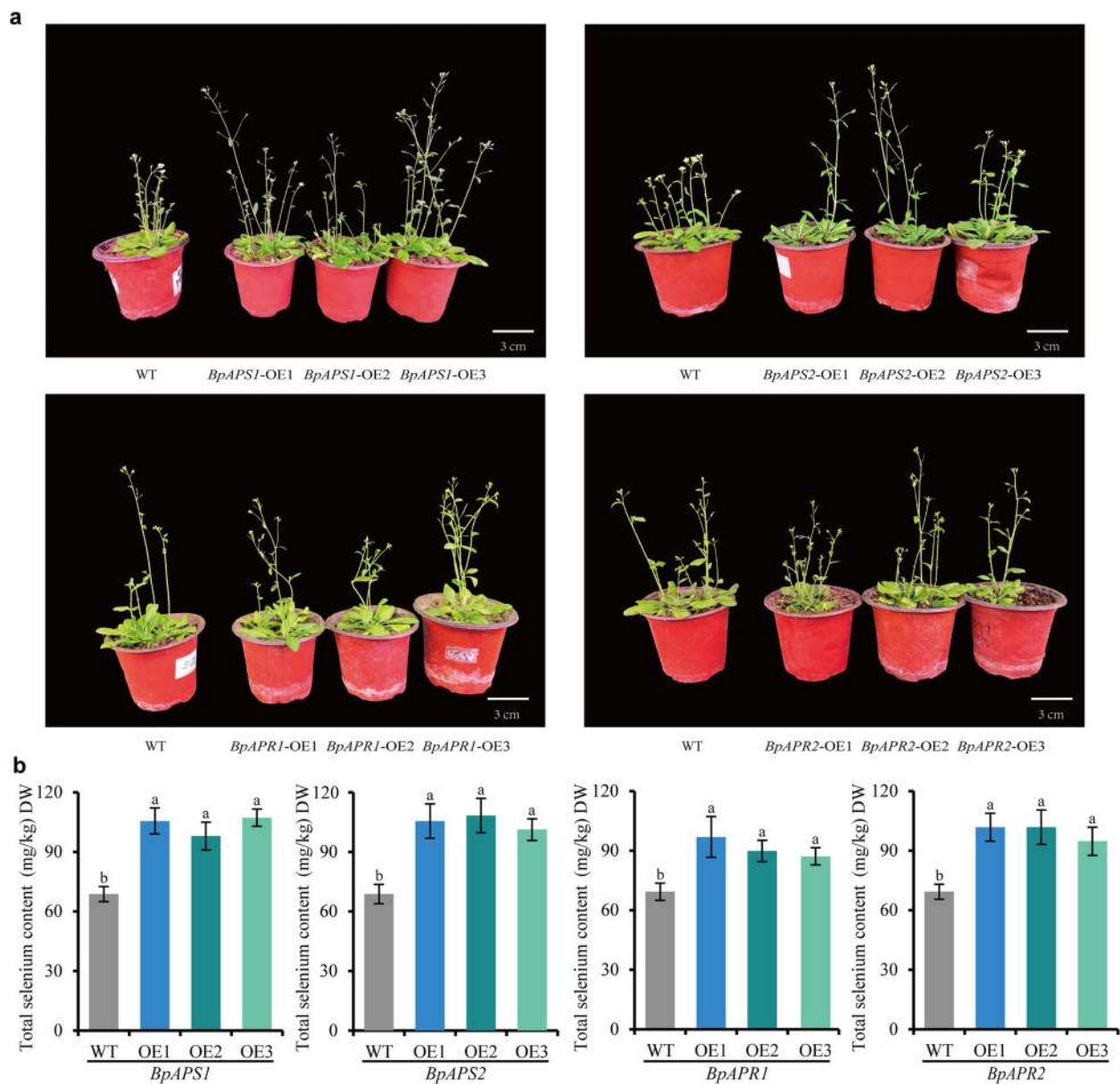


Fig. 5 Selenium accumulation and expression of selenium metabolism-related genes in transgenic *Arabidopsis thaliana* overexpressing ATP sulfurylase 1 (*BpAPS*) and adenosine 5'-phosphosulfate reductase 1 (*BpAPR*) genes of *Broussonetia papyrifera*. **a** Total selenium content in wild-type (WT) and homozygous T3 transgenic lines overexpressing *BpAPS1*, *BpAPS2*, *BpAPR1*, and *BpAPR2* under 0.4 mM Na_2SeO_4 treatment. *BpAPS*-OE, *BpAPS* overexpressing lines; *BpAPR*-OE, *BpAPR* overexpressing lines. **b** Selenium content comparison across different transgenic lines. DW, dry weight. Different letters indicate significant differences based on Duncan's multiple range test ($P < 0.05$)

APR-overexpressing lines, indicating a shared volatilization-based detoxification mechanism (Fig. 7). These distinct but overlapping transcriptional responses demonstrate that the overexpression of *BpAPS* and *BpAPR* genes coordinately enhances Se activation, reduction, methylation, and volatilization pathways, thereby improving Se assimilation, transformation in transgenic plants.

Discussion

Organ-specific growth responses and selenium partitioning under an elevated supply of selenium

Se treatment induced organ-specific alterations in the growth and Se distribution of *B. papyrifera*. Under high-Se conditions, growth inhibition was most pronounced in aerial tissues, with significant reductions in leaf expansion and shoot elongation. In contrast, the

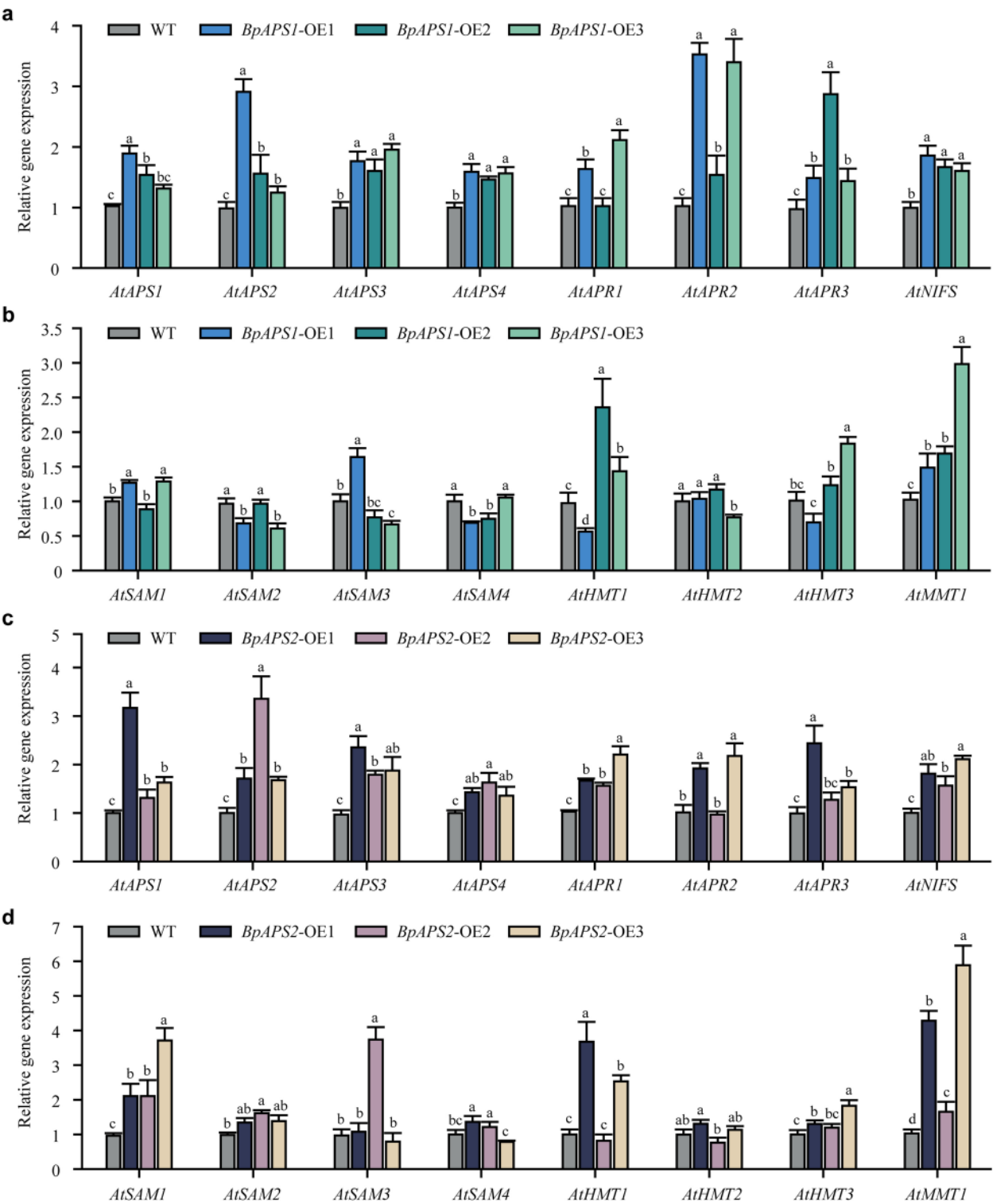


Fig. 6 Relative expression of selenium metabolism-related genes in *Arabidopsis thaliana* overexpressing *Broussonetia papyrifera* ATP sulfurylase 1 (*BpAPS1*; **a, b**) and *BpAPS2* (**c, d**). WT, wild-type; *BpAPS*-OE, *BpAPS* overexpressing lines; *APR*, adenosine 5'-phosphosulfate reductase; *NIFS*, nitrogen fixation S-like cysteine desulfurase; *SAM*, S-adenosylmethionine; *HMT*, Homocysteine S-methyltransferase; *MMT*, methylselenol methyltransferase. Different letters represent significant differences according to Duncan's multiple range test ($P < 0.05$)

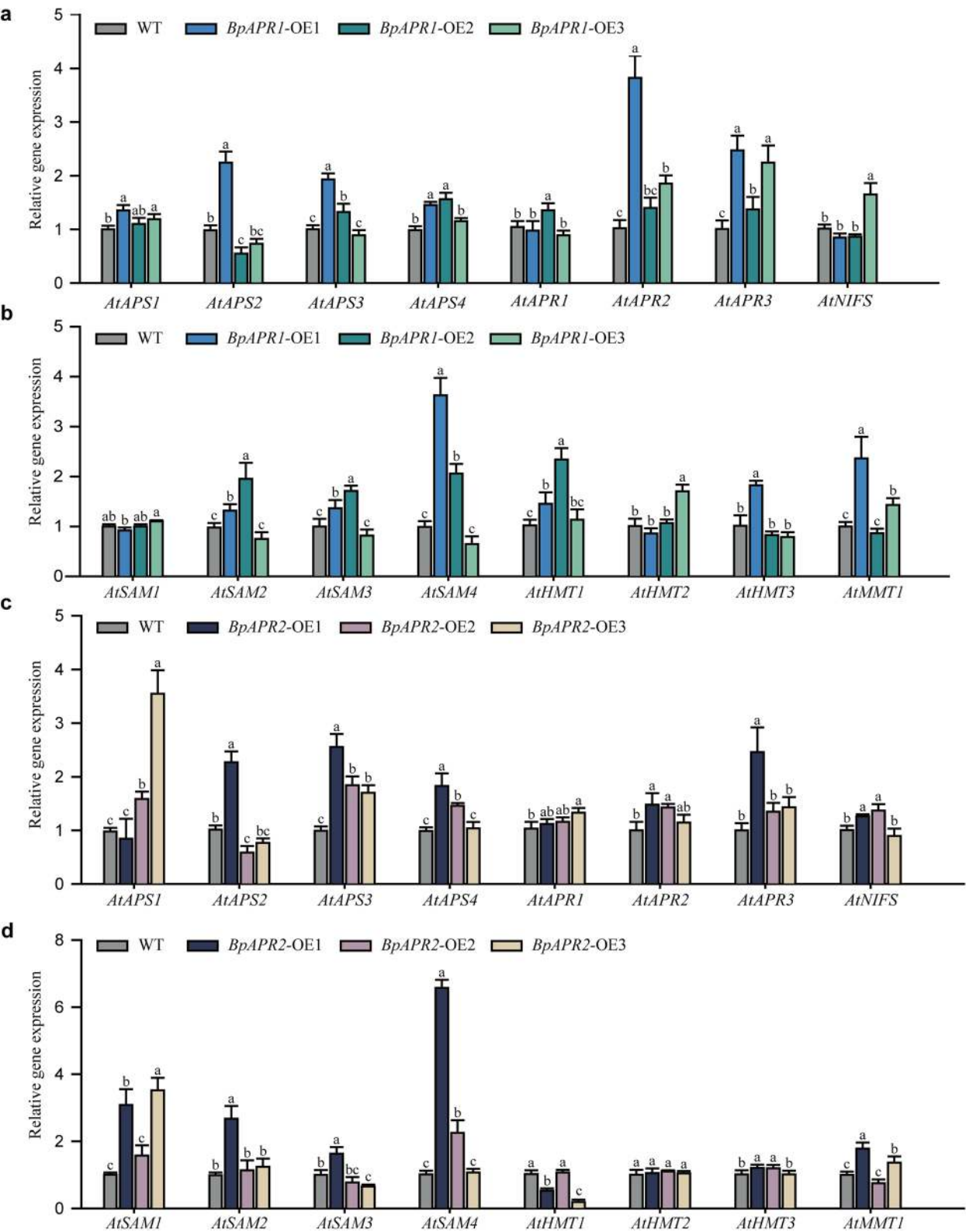


Fig. 7 Relative expression of selenium metabolism-related genes in *Arabidopsis thaliana* overexpressing *Broussonetia papyrifera* adenosine 5'-phosphosulfate reductase 1 (*BpAPR1*; **a, b**) and *BpAPR2* (**c, d**). WT, wild-type; *BpAPR*-OE, *BpAPR* overexpressing lines; APS, ATP sulfurylase; NIFS, nitrogen fixation S-like cysteine desulfurase; SAM, S-adenosylmethionine; HMT, Homocysteine S-methyltransferase; MMT, methylselenol methyltransferase. Different letters represent significant differences according to Duncan's multiple range test ($P < 0.05$)

roots accumulated higher levels of Se but exhibited less pronounced growth suppression. This pattern suggests differential sensitivity of plant organs to Se stress (Chen et al. 2024).

The leaves exhibited preferential Se accumulation and greater growth inhibition, indicating that aerial tissues function as primary Se sinks and sites of stress perception. Consequently, growth inhibition under high-Se conditions reflects physiological adaptation rather than acute toxicity. Roots and stems showed relatively stable growth, suggesting that plants preserve structural integrity and transport capacity under Se stress. Overall, *B. papyrifera* responded to Se in a dose-dependent manner, with low Se levels promoting growth and high levels inhibiting it. This biphasic response aligns with the hormesis model, providing a physiological foundation for subsequent transcriptional regulation.

Conserved and dose-dependent functional divergence of *BpAPS* and *BpAPR* genes in selenium metabolism

Comparative studies have shown that APS and APR isoforms, including FcAPS2 and FcAPR1, play conserved roles in plastid-localized sulfate and Se activation and reduction (Takahashi et al. 2011, 2014). In line with these findings, *BpAPS* and *BpAPR* clustered with homologs from *M. alba*, *P. trichocarpa*, and *A. thaliana*, supporting their conserved roles in sulfate and Se metabolism. Despite this conservation, their dose- and tissue-dependent expression variations under Se treatment indicate functional specialization. *BpAPS1* was predominantly induced under low-Se conditions, suggesting its primary role in basal Se assimilation. In contrast, *BpAPS2* was significantly upregulated at high Se concentrations. Coupled with the presence of stress-responsive *cis*-elements in its promoter, this pattern suggests that *BpAPS2* is likely involved in stress-related regulatory signaling, rather than Se assimilation alone (El Mehdawi et al. 2018). The functional differences between *BpAPR1* and *BpAPR2* further emphasize the fine-tuned regulation of Se reduction (Freeman et al. 2010). This functional diversification likely represents an adaptive strategy that allows *B. papyrifera* to dynamically balance Se uptake, assimilation, and detoxification under fluctuating environmental conditions (Schiavon et al. 2015; Dall'Acqua et al. 2019).

In this context, the strong induction of *BpAPS2* likely reflects a stress-responsive transcriptional adjustment to elevated Se exposure. Together with the stress-induced activation of *BpAPS2* under high-Se conditions, these findings suggest that *APS* and *APR* genes not only facilitate Se assimilation but also activate downstream tolerance and detoxification mechanisms when Se availability exceeds optimal levels (Chauhan et al. 2020). Differential

responses to selenate and selenite further highlight specialized regulatory networks involving *BpAPS* and *BpAPR*, supporting a hormetic model in which low Se promotes growth, whereas higher levels trigger stress mitigation and detoxification pathways (Chen et al. 2022; Zhu et al. 2025).

Overexpression of *BpAPS* and *BpAPR* enhances Se assimilation and tolerance in *Arabidopsis*

Overexpression of *BpAPS1* and *BpAPS2* primarily enhances upstream Se activation, reflected by the coordinated upregulation of endogenous *AtAPS* isoforms and the induction of *AtAPR*, which together increase the metabolic flux from selenate toward reductive assimilation (Pilon-Smits et al. 1999; Zhang and Chu 2022; Chao et al. 2022). In contrast, overexpression of *BpAPR1* and *BpAPR2* directly enhances Se reduction and downstream processing. These lines strongly upregulate *AtAPR* and detoxification-related genes, including *AtSAM*, *AtHMT*, and particularly *AtMMT1*, which facilitate the conversion of reduced Se into less toxic or volatile forms (Li et al. 2008; Ahmed et al. 2019). Quantitative biomass analysis further confirmed the phenotypic observations. Together, these results support a regulatory model in which *APS* serves as the primary driver of Se entry and activation, while *APR* functions as a critical control point linking Se reduction to downstream detoxification pathways.

Integrated regulation of selenium assimilation and homeostasis by *BpAPS* and *BpAPR*

Confocal microscopy confirmed that all *BpAPS* and *BpAPR* proteins were localized to chloroplasts, consistent with the localization of sulfur assimilation enzymes and Se metabolism in plastids (Schiavon et al. 2015). The presence of these enzymes in chloroplasts suggests their direct involvement in converting Na_2SeO_4 to Na_2SeO_3 , followed by the reduction to seleno-amino acids such as SeCys and SeMet via the reductive assimilation pathway (Schiavon and Pilon-Smits 2017). This localization also implies participation in generating redox intermediates (e.g., GSH and NADPH), critical cofactors for Se metabolism (Schiavon et al. 2020; Nogueira et al. 2021).

Additionally, plastid compartmentalization may provide a protective advantage by limiting the cytosolic exposure to reactive Se intermediates, thus reducing oxidative stress (Pottosin and Sergey Shabala 2016). Se methylation and volatilization within chloroplasts contribute to Se detoxification and homeostasis through the generation of volatile organo-Se compounds (Van Hoewyk et al. 2005; Trippe and Pilon-Smits 2021; Jagot et al. 2025). In this plastid-centered metabolic context, *BpAPS2* may function as a regulatory node that integrates

Se availability and stress signals, contributing to feedback control over Se assimilation and tolerance rather than simply promoting Se accumulation. Overall, the chloroplast localization of BpAPS and BpAPR proteins suggests their functional integration not only in primary Se assimilation but also in downstream detoxification processes, potentially coordinating with plastid-localized methylation and volatilization machinery to regulate Se tolerance and homeostasis.

Conclusions

In this study, four Se assimilation-related genes from *B. papyrifera* were cloned and characterized, focusing on their sequence features, tissue-specific expression, sub-cellular localization, and biological functions. Promoter and phylogenetic analyses indicated their involvement in stress responses and strong evolutionary conservation with homologs from *M. alba*. Transient expression assays confirmed that all four proteins localized to chloroplasts, highlighting their role in chloroplast-mediated Se metabolism. Exogenous Se treatments induced concentration-dependent effects on plant growth and Se accumulation, with Na₂SeO₄ promoting greater uptake efficiency. The expression of *BpAPS1*, *BpAPR1*, and *BpAPR2* correlated with tissue Se levels, while *BpAPS2* exhibited a distinct expression pattern, suggesting a specialized function. Transgenic *Arabidopsis* lines overexpressing these genes accumulated more Se, with enhanced expression of key Se metabolism-related genes, increasing Se uptake, assimilation, volatilization, and ultimately tolerance. These findings lay the molecular foundation for Se biofortification in woody forage species.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1007/s44281-026-00104-y>.

Supplementary Material 1: Figure S1. Molecular identification of T1-generation transgenic plants and phenotypic analysis of T1/T3-generation transgenic *Arabidopsis thaliana* plants. WT, wild-type; *BpAPS*-OE, *Broussonetia pendula* ATP sulfurylase-overexpressing lines; *BpAPR*-OE, adenosine 5'-phosphosulfate reductase-overexpressing lines.

Supplementary Material 2: Table S1. ATP sulfurylase (APS) protein sequences used in phylogenetic analysis. Table S2. Adenosine 5'-phosphosulfate reductase 1 (APR) protein sequences used in phylogenetic analysis. Table S3. Primer sequences for cloning the *Broussonetia papyrifera* APS (*BpAPS*) and *BpAPR* genes. Table S4. Primer sequences for gene amplification. Table S5. Primer sequences used for the qRT-PCR analysis of Se-related genes in *Arabidopsis thaliana*. MMT, Methylselenol Methyltransferase; HMT, Homocysteine S-methyltransferase; NifS, Nitrogen Fixation S-like Cysteine Desulfurase; SAM, S-adenosylmethionine. Table S6. Comparative physicochemical and structural properties of *BpAPS1*, *BpAPS2*, *BpAPR1*, and *BpAPR2*.

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Authors' contributions

YSX: conceptualization, software writing—original draft; CXQ: data curation, formal analysis; MX and JXS: conducting a research, investigation; QWC and JRZ: data collection, visualization; HJG: writing—review and editing; WWZ: supervision, funding acquisition; FX: conceptualization, funding acquisition, writing—review and editing, supervision, and validation.

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Data availability

The data supporting the findings of this work are available within the paper and its supplementary files. The datasets generated and analyzed during the current study is available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

Authors declare that they have no competing interests.

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