

Overexpression of foxtail millet (*Setaria italica*) amino acid permease 9 (SiAAP9) inhibits the growth in transgenic Arabidopsis

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Abstract

Amino acid permeases (AAPs) transporters play a vital role in facilitating the long-distance transportation of amino acids from the source to the sink in plants. Numerous studies have been conducted on *Arabidopsis* and rice, while the research on foxtail millet remains relatively scarce. In this study, we discovered that *SiAAP9* exhibits two transcripts. The expression patterns of the two transcripts of *SiAAP9* were generally consistent and both transcripts were induced by NO_3^- . Overexpression of *SiAAP9L* and *SiAAP9S* in *Arabidopsis* inhibited plant growth and seed size, however, *SiAAP9* can transport more amino acids into seeds. Moreover, the tolerance of WT and *SiAAP9-OX* transgenic *Arabidopsis* to amino acids demonstrated that *SiAAP9-OX* transgenic *Arabidopsis* lines exhibit tolerance to high concentrations of glutamate (Glu) and histidine (His). The high expression level of *SiAAP9* makes its encoded protein not only localized on the plasma membrane, but also possibly localized on various organelles. Additionally, we also observed that sequence deletion weakened the sensitivity of *SiAAP9* to Brefeldin A (BFA) and *SiAAP9* has ectopic localization on endoplasmic reticulum (ER). Protoplast amino acid uptake experiments indicated that *SiAAP9* is capable of facilitating increased transport of Glu into foxtail millet cells. Taken together, the functions of two *SiAAP9* transcripts are largely functional similar, but the degree of colocalization of *SiAAP9L* and BFA compartment is significantly higher than that of *SiAAP9S*. Our research may provide a candidate gene to improve foxtail millet nutritional quality in breeding.

Key Message

The two transcripts of *SiAAP9* that facilitated amino acids uptake and showed similar biological functions in *Arabidopsis*.

Introduction

Amino acids are crucial nutrient for the growth and development of plants (Gu et al. 2018; Marmagne et al. 2022). It is primarily transported within plants through the xylem and phloem (Fischer et al. 1998; Tegeder and Masclaux-Daubresse 2018). The long-distance movement of amino acids relies on specific transporters on the plasma membrane for loading and unloading (Dinkeloo et al. 2018; Tegeder and Masclaux-Daubresse 2018; Yao et al. 2020). Recent studies have highlighted the significant role of amino acid transporters (AATs) in the distribution of nitrogen from source to sink (Yang et al. 2020). Additionally, AATs contribute to the regulation of crop yield (Zhang et al. 2015), crop nutritional quality (Peng et al. 2014), seed development (Schmidt et al. 2007), carbon and nitrogen metabolism (Grant et al. 2021) and stress resistance (Tian et al. 2020).

Biographical information reveals that AAP (amino acid permease) is categorized under a subfamily of the AAAP family within the AAT superfamily (Ma et al. 2016; Wu et al. 2015). AAPs have been extensively studied in *Arabidopsis* and other crops. In *Arabidopsis*, AtAAP1 is involved in regulating the entry of amino acids into developing embryos and promoting protein synthesis in seeds (Chen et al. 2021; Sanders et al. 2009). Under varying nitrogen conditions, the *Ataap2* mutant exhibits increased allocation

of nitrogen to the leaves, enhancing their photosynthetic capacity (Perchlik and Tegeder 2018). Additionally, AtAAP3 and AtAAP6 play a crucial role in transferring amino acids into reservoir organs during pathogen infection (Sonawala et al. 2018). In rice, transporters such as OsAAP1, OsAAP4, and OsAAP15 have been shown to increase rice tillering and grain yield by regulating the distribution of neutral amino acids (Fang et al. 2021; Ji et al. 2020; Yang et al. 2023), whereas OsAAP3 and OsAAP5 play an opposite role (Lu et al. 2018; Wang et al. 2019). Recent research has demonstrated that the use of the CRISPR/Cas9 system to knock out *OsAAP6* and *OsAAP10* can reduce grain protein content (GPC) and improve eating and cooking quality (ECQ) (Wang et al. 2020). Moreover, overexpression of *OsAAP6* in rice plants increases the protein content of rice grains, especially under nitrogen rich conditions (Peng et al. 2014). ZmAAP6 has also been identified as a crucial positive regulator of maize protein content and nutritional quality (Wang et al. 2022). *GmAAP6a* transgenic soybean exhibits enhanced nitrogen transport to seeds, resulting in higher biological yield under low nitrogen conditions (Liu et al. 2020). In potatoes, the *StAAP1* antisense line inhibits the transport of amino acids from source organs to the phloem, leading to a significant reduction of amino acids in tuber (Koch et al. 2003). TaAAP3, TaATLa2 and TaATLb13 are involved in the response to drought and high-temperature stresses by regulating amino acid distribution (Tian et al. 2019; Tian et al. 2020). However, regarding the research on foxtail millet AAPs, it has been reported that *SiAAP9* is highly expressed in foxtail millet immature spikelets and seeds only through Preliminary bioinformatics and RNA-seq analysis (Yang et al. 2021), but there is no in-depth analysis of the functions of *SiAAP9*. Foxtail millet is a traditional crop in China with nutritional value (Xu et al. 2020b), including higher protein and crude fiber content (Sharma and Niranjana 2018), as well as nine essential amino acids required by the human body (Sachdev et al. 2021). Consuming foxtail millet has been shown to reduce the risk of diabetes (Chen et al. 2017; Wang et al. 2023).

In this study, we used *SiAAP9* T₃ generation homozygous transgenic *Arabidopsis* material to study the biological functions of *SiAAP9L* and *SiAAP9S*. *SiAAP9S* lacks a large loop structure compared to *SiAAP9L* on Exon4. *SiAAP9* is constitutively expressed and induced by NO₃⁻. Overexpression of *SiAAP9* inhibited the growth and development of transgenic *Arabidopsis* vegetative organs, but significantly increased the content of amino acids in seeds. Specific amino acid substrate by *SiAAP9* transport has shown *SiAAP9-OX* transgenic *Arabidopsis* lines exhibit tolerance to high concentrations of Glu and His. The transformation experiment of foxtail millet protoplasts further confirmed that *SiAAP9* can transport more Glu into foxtail millet cells. Overexpression of *SiAAP9* resulted in abnormal protein localization, and the vesicle transport pathway indicated that sequence deletion reduced sensitivity to BFA and excessive *SiAAP9* protein colocalized with ER. In summary, the two transcripts of *SiAAP9* inhibit the growth and development of transgenic *Arabidopsis*, but promote the absorption of amino acids.

Materials and methods

Plasmids construction and plant transformation

The DNA sequence of *SiAAP9* (*Seita.5G401000.1*) and its encoded protein

sequence were obtained from the Phytozome website (<https://phytozome.jgi.doe.gov/pz/portal.html>). Transmembrane domains of SiAAP9 were predicted using the web-based TMHMM Server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). To construct the *SiAAP9-OE* vector, a 1419 bp *SiAAP9* coding sequence was amplified using the primers 5'-CACGGGGGACGAGCTCGGTACCATGGACGTGGAGAAGATCGAGAGGA-3' and 5'-GTGGCGCGCCGGGCCCTCTAGAGAGCTGCGTCTGGAAGATGGTG-3' and inserted downstream of the 35S promoter in p35S-MCS-EGFP-6×His-35S-Hyg (a binary vector) using KpnI and XbaI restriction enzymes. Cloning *SiAAP9* coding sequence using 5'-TACCGTCGACGAGCTAAGCTTGATGGACGTGGAGAAGATCGAGAGGA-3' and 5'-GGGAAATTCGAGCTCGGTACCTCAGAGCTGCGTCTGGAAGATGGTG-3' primers to link to the HindIII and KpnI restriction enzymes of binary vector pCUN-NHF from Aying Zhang laboratory (Yan et al. 2022) to construct a plasmid for transforming foxtail millet protoplasts, mainly used to study the transport of Glu. To analyze the expression pattern of *SiAAP9*, *SiAAP9* Promoter::GUS vector was constructed, a 2737 bp *SiAAP9* promoter (upstream of the start codon ATG) was cloned using the primers 5'-TGTCAAACACTGATAGTTTAAACCTCGCTTGTGTATGCGTGCTTCG-3' and 5'-CCCGGGGATCGATCCTCTAGACGCTGAAAGCTGTCTGTTGCAGCT-3' and inserted into the CCDB position of *PAL47* (CCDB-GUSA-Hyg) vector with Pme and XbaI.

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used for all experiments and as the genetic background for corresponding plant transformations. To ensure the authenticity of the fusion constructs, DNA sequence was performed prior to transformation into *Agrobacterium* strain GV3101 for stable transformation of *Arabidopsis*. The *Agrobacterium*-mediated floral dipping method was employed for *Arabidopsis* transformation. Transformants were firstly selected on 1/2 MS medium supplemented with 1/2000 (50 mg/mL) hygromycin B (Coolaber, 31282-04-9) and then selected positive seedlings were transferred to vermiculite to pass down generations. *LOX-1#*, *LOX-2#*, *SOX-1#* and *SOX-2#* lines were detected for their expression levels through RT-qPCR and western blot analysis. T₃ homozygous *SiAAP9-OX* transgenic *Arabidopsis* lines were used for phenotype analysis. *Arabidopsis* plants were cultivated in a greenhouse under long-day conditions, with a photoperiod of 16 hours of light and 8 hours of darkness, at a temperature range of 18–22°C.

Conventional variety foxtail millet Jingu 21 seedlings cultured in 7 mM KNO₃ or 0 mM KNO₃ solution for 7 days were grown in 25–30°C with 16 hours of light and 8 hours of darkness.

Histochemical GUS staining assay

For the tissue-specific expression assay, three independent *SiAAP9 pro::GUS Arabidopsis* lines were generated, various tissues of *SiAAP9 pro::GUS* transgenic lines were incubated in staining buffer (32 mM Na₂HPO₄, 18 mM KH₂PO₄, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, Triton X-100 and 1 mg/mL X-Gluc) at 37°C overnight. After discoloring by incubation in a solution of 70% ethanol, the stained samples were observed using Olympus BX51 microscope.

RNA isolation and RT-qPCR analysis

The seeds of Jingu 21 were sterilized with 0.1% mercuric chloride solution for 10 min and were cultured in a bean sprout box with 7 mM KNO₃ and 0 mM KNO₃ for 7 days respectively, changing the nutrient solution every three days. And then 7-day-seedlings were transferred to 0 mM KNO₃ or 7 mM KNO₃ solution for 10 min, 30 min and 1 hour respectively to explore whether *SiAAP9* is induced by NO₃⁻. Total RNA was extracted using Trizol reagent according to the manufacturer's instructions (TransGen Biotech, ER501-01-V2). Weigh 100 mg of seedlings as a repeat and RNA was extracted. First strand cDNA were synthesized using 1 µg of total RNA extracted from each sample using reverse transcriptase (ABM, G592). RT-qPCR was performed in a 10 µL reaction volume containing 4 µL 2 × Realtime PCR Super mix (Mei5bio, MF004-10), 1 µL cDNA solution and 5 µL specific primers (2 µM) under the following conditions: 95°C for 5 min (1 cycle), 95°C for 20 s, 55°C for 20 s and 72°C for 20 s (40 cycles), and 72°C for 5 s (1 cycle) using real-time fluorescence quantitative PCR instrument (American Bole Bio-Rad CFX96). *Siactin2* (*Seita.8G043100*) was used as an internal control for normalization. Three biological and three technical replicates were analyzed for RT-qPCR experiments. The fluorescent quantitative primers for *SiAAP9*, *SiAAP9L*, *SiAAP9S* and *Siactin2* are designed as shown in Supplementary Table 1.

Nitrogen and amino acid treatment assay

To analyze the response of WT and *SiAAP9-OX* transgenic *Arabidopsis* lines to KNO₃ and KCl conditions, seeds were sterilized with 70% ethanol and grown on MGRl medium containing multiple trace elements, the concentration of NO₃⁻ was set to 0 mM and 7 mM. 4-day-old seedlings grown on 7 mM KNO₃ and 7 mM KCl medium were transferred to 7 mM KCl and 7 mM KNO₃ medium for 8 days respectively. The primary root length and root length variation of 20 seedlings were measured using Image J software and fresh weight was weighed. To investigate the tolerance of WT and *SiAAP9-OX* transgenic *Arabidopsis* lines to 20 common amino acids (Tyrosine is soluble in HCl, so we did not study it). All amino acids were purchased from Coolaber, BeiJing. Amino acids include L-Glycine (Coolaber, 56-40-6), L-Alanine (Coolaber, 56-41-7), L-Valine (Coolaber, 72-18-4), L-Leucine (Coolaber, 61-90-5), L-Isoleucine (Coolaber, 73-32-5), L-Methionine (Coolaber, 63-68-3), L-Proline (Coolaber, 147-85-3), L-Tryptophan (Coolaber, 73-22-3), L-Serine (Coolaber, 56-45-1), L-Cysteine (Coolaber, 52-90-4), L-Phenylalanine (Coolaber, 63-91-2), L-Asparagine (Coolaber, 70-47-3), L-Glutamine (Coolaber, 56-85-9), L-Threonine (Coolaber, 72-19-5), L-Aspartic acid (Coolaber, 56-84-8), L-Glutamic acid (Coolaber, 56-86-0), L-Lysine monohydrate (Coolaber, 39665-12-8), L-Arginine (Coolaber, 74-79-3), L-Histidine (Coolaber, 71-00-1). WT and *SiAAP9-OX* transgenic *Arabidopsis* lines were cultured on 1/2 MS medium containing different types and concentration gradient of amino acids for 12 days. We further explored the tolerance of *SiAAP9* transgenic *Arabidopsis* to amino acids by using amino acids with significant phenotypic differences as the sole nitrogen source to cultivate seedlings for 12 days.

Subcellular localization under different NO₃⁻ conditions

To explore the localization of SiAAP9 under 7 mM NO_3^- and 0 mM NO_3^- , two transcripts coding sequence were fused into vectors with GFP tag driven by the *CaMV35S* promoter. Transgenic T_3 generation *Arabidopsis* seeds were sterilized and incubated for four days, stained by amphiphilic styryl dye FM4-64 reagent and photographed roots using a laser confocal microscope. Under different NO_3^- conditions, image J software was used to calculate the fluorescence intensity of GFP in forty meristematic cells to determine the difference between 7 mM NO_3^- and 0 mM NO_3^- conditions.

Observation of SiAAP9 localization by reagent treatment

Use FM4-64 (Coolaber, CD4673) to stain plant materials while observing cell contours and endocytic transport. Stained with 4 μM FM4-64 for 1 min and located on the plasma membrane (PM). Stained for 5 minutes and transfer seedlings to 1/2 MS medium for 15 min, 30 min and 2–3 h respectively, and then will be observed to be located on Trans Golgi Network/Early Endosome (TGN/EE), Prevacuolar Compartment (PVC) and vacuolar membranes. Two T_3 lines of the two transcripts were stained separately and laser confocal scanning was performed to observe the degree of colocalization. In addition to using FM4-64 staining to observe the location, ER Tracker (Beyotime, C1041S) was used to observe whether SiAAP9 was retained in ER, which leads to the inability to move to the appropriate location to perform functions. For BFA treatment, 4-day seedlings were treated in liquid 1/2 MS medium plus 50 μM BFA (Coolaber, 20350-15-6) and 4 μM FM4-64 for 50 minutes. In addition, 33 μM WM (Wortmannin) (Coolaber, 19545-26-7) and 4 μM FM4-64 were added to 1/2 MS liquid medium for 1 h. The seedlings were washed three times and then were observed using a confocal laser scanning microscope.

Western Blot

Plant tissues were crushed after liquid nitrogen freezing and added 100 μL 2[×] sample buffer (0.1 mg/mL BPB, 40 mg/mL SDS, 20% (v/v) glycerol, 0.13 M Tris-HCl). The sample was shaken thoroughly and then boiled in 90°C for 3–5 min. After centrifugation at 12000 rpm for 10 min, proteins were separated by 8% (w/v) SDS-PAGE and transferred to a Nitrocellulose (NC) membrane in a wet transfer system. A 1:5000 dilution primary antibody Anti-GFP Mouse mAb (TransGen Biotech, D037), anti-actin (Bioss, bsm-33128M) and 1:5000 dilution secondary antibody Goat Anti-Mouse IgG, HRP (TransGen Biotech, T156) were added to bind to specific proteins. After 2–3 h of incubation, the developer solution Supper ECL Immunoblotting Substrate (Coolaber, SL1350) was added and the membranes were photographed with a protein imager (Vilber, Fusion FX6.EDGE).

Protoplast preparation and transformation

Foxtail millet protoplast isolation method was modified according to the wheat and rice protoplast (Shan et al. 2014). The shoot tissues of Jingu 21 seedlings that have grown for 7–14 days were cut with 0.5 mm to 0.4 M mannitol for 10 min in a dark condition. Then plant materials were transferred to the 15 mL enzyme solution (20 mM MES (pH 5.7), 0.4 M mannitol, 20 mM KCl, 1.5% Cellulase R10, 0.75% macerozyme, 10 mM CaCl_2 , 0.1% BSA), vacuumized for 30 min at 380 ~ 508 mm Hg, and was immediately incubated for 5–6 hours in the dark under 60–80 rpm. After enzymatic hydrolysis, W5 (154

mM NaCl, 125 mM CaCl₂, 5 mM KCl, 2 mM MES) solution was added to release protoplasts. The supernatant obtained through filtration was centrifuged for 3 min at 250 g, and then resuspended with an appropriate volume of W5. After 30 min of ice bath, sediment suspended by MMG (0.4 M mannitol, 15 mM MgCl₂, 4 mM MES). Finally, 10 µg of empty vector, *OE-SiAAP9L* and *OE-SiAAP9S* were added to the foxtail millet protoplasts and incubated in a 24 hole plate for 16 hours at 25 °C under dark conditions.

Protoplast amino acid uptake assay

Amino acids labeled with FITC (Glu-FITC) were synthesized by Yuan Peptide Biotechnology Company, Nanjing, China. After dark incubation, the protoplasts were added with FITC labeled Glu to the final concentration of 0.1 mM in the dark for 4 hours and were centrifuged at 250 g for 3 min, the supernatant was carefully removed and the protoplasts were suspended with 1 mL WI (4 mM MES, 0.5 M mannitol, 20 mM KCl) solution for three times to remove free amino acids. Finally, the fluorescence signals were observed using a confocal laser scanning microscope.

Statistical analysis

The data were analyzed by one-way ANOVA followed by Student's t and Duncan test, with the following significance levels: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ or lowercase letters at $p < 0.05$.

Results

SiAAP9 exhibits two alternative splicing (AS) events.

Previous study (Yang et al. 2021) has shown that *SiAAP9* may highly expressed in immature spikelets and seeds during the filling stage. We supposed that *SiAAP9* may play an important role in regulating seed development. At first, *SiAAP9* was cloned using cDNA templates of Jingu 21 (Fig. 1A). Surprisingly, the bands of the colony PCR that linked *SiAAP9* coding sequence to the CV16 plasmid cloning vector were not on the same horizontal line (Fig. 1B). Through sequence alignment, it was found that *SiAAP9* lacked a sequence on Exon4 (Fig. 1C, supplementary Fig. S1A). Therefore, we labeled the long sequence as *SiAAP9L* and the short sequence as *SiAAP9S*. Additionally, AAP transporters have previously been reported as plasma membrane transporters in *Arabidopsis* and rice (Ji et al. 2020; Santiago and Tegeder 2016), we predicted SiAAP9 transmembrane domain and protein tertiary structure. Notably, we observed that the protein encoded by the long transcript exhibited an extra extensive loop structure spanning amino acids 200 to 250 in contrast to the protein encoded by the short transcript (supplementary Fig. S1C, D, E, F).

The expression patterns analysis of *SiAAP9*.

To detect the complete, long and short transcripts of *SiAAP9*, specific primers were designed (Fig. 2A). RT-qPCR analysis of different tissues of Jingu 21 showed that the expression levels of the two transcripts were higher in shoot and lower in leaves, flowers and grains (Fig. 2B-D). In order to investigate whether *SiAAP9* is induced by short-term NO₃⁻ treatment, we performed RT-qPCR analysis and found that after 1

hour of NO_3^- addition, the expression level of *SiAAP9* two transcripts showed a significant increase, while there was no significant change after 1 hour of NO_3^- reduction (Fig. 2E-J). This suggests that *SiAAP9* is induced by short-term NO_3^- exposure. Additionally, transgenic *Arabidopsis* plants were used to observe the expression of *SiAAP9*. The results of GUS staining revealed that *SiAAP9* is expressed in all tissues throughout developmental stages, including seedlings (supplementary Fig. S2A), leaves (supplementary Fig. S2B, D), stomata (supplementary Fig. S2E), flower organs (supplementary Fig. S2F), pistil (supplementary Fig. S2G), root (supplementary Fig. S2H), stamen (supplementary Fig. S2I) and developing seeds (supplementary Fig. S2J). However, *SiAAP9* showed low expression in the root tip (supplementary Fig. S2C).

***SiAAP9* inhibits growth and development in transgenic *Arabidopsis*.**

To further investigate the function of *SiAAP9*, we generated transgenic plants by overexpressing *SiAAP9* in *Arabidopsis* using the 35S promoter. Long and short transcripts each selected two independent T_3 homozygous overexpressing lines, namely *LOX-1#*, *LOX-2#*, *SOX-1#* and *SOX-2#*. These lines were identified through RT-qPCR and Western Blot analysis (supplementary Fig. S3). For subsequent experiments, we specifically selected two lines with significantly different expression levels in *SiAAP9L*. Throughout the entire growth and development period of the transgenic plants, *LOX-1#* showed no significant difference compared to the WT in agronomic traits, the other lines exhibited a significant decrease in growth compared to the WT. The primary root length at 7 days of *LOX-1#* increased by 4.3%, while the other three lines decreased by 5.0%, 5.9% and 3.4% respectively compared to WT. There was no significant difference in the rosette leaves area at 3 weeks between *LOX-1#* and WT, while the other three lines also significantly decreased by 56%, 16.8% and 20.1%, respectively. And plant height at 4-8 weeks displayed obviously dwarfism (Fig. 3). The size and weight of grains have a close relationship with crop yield and nutritional quality (Hori and Sun 2022; Li et al. 2022). Additionally, the seed size and 1000-seed weight were also suppressed in *SiAAP9* transgenic plants compared to those in Col-0, in which *LOX-2#* has a more significant reduction compared to other lines (Fig. 4A-D), indicating that *SiAAP9* negatively regulates the size and weight of seeds. To further investigate whether overexpression of *SiAAP9* affects the content of amino acids in seeds, we measured the content of 20 amino acids in seeds and found that the amino acid content in seeds of *SiAAP9-OX* transgenic *Arabidopsis* lines was significantly higher than that in WT, except for Arg, Asn and Asp. Notably, *LOX-2#* and *SOX-2#* have significantly higher content of most amino acids than WT (supplementary Fig. S4). These results indicate that *SiAAP9* has an inhibitory effect on the development of vegetative organs in *SiAAP9-OX* transgenic *Arabidopsis* lines, but increases the amino acid content in the seeds, may increase the nutritional quality potentially.

The effect of *SiAAP9* on primary root growth is determined by its expression level, regardless of nitrogen availability.

Previous studies have shown that under nitrogen sufficient conditions, there is no significant difference in the primary root length between overexpressing *GmAAP6a* and WT. However, under nitrogen free conditions, the primary root length of overexpressing *GmAAP6a* is significantly longer than WT (Liu et

al. 2020). Hence, we aim to explore the impact of varying nitrogen levels on the biological function of *SiAAP9*.

In this study, we investigated the response of *SiAAP9* to NO_3^- and NO_3^- free. When exposed to NO_3^- free stress, *SiAAP9-OX* transgenic *Arabidopsis* lines have smaller aerial parts and fewer and shorter lateral roots in the primary root. Furthermore, after being cultured on a nitrogen rich medium for 4 days and then transferred to NO_3^- free medium for 8 days, we observed that the primary root length, root variation length and fresh weight of *LOX-1#* were promoted slightly than WT, while *LOX-2#* and *SOX-1#* lines were significantly reduced than WT. However, *SOX-2#* showed no significant changes (Fig. 5). Interestingly, the seedlings transferred from 0 mM NO_3^- medium for 4 days to 7 mM NO_3^- medium for 8 days showed the same significant change trend (supplementary Fig. S5). Combined with the research results in Fig. 3 and Fig. 4, both indicate that low expression levels of *SiAAP9* had no significant effect on the growth and development in *SiAAP9-OX* transgenic *Arabidopsis* lines, while high expression levels of *SiAAP9* had a significant inhibitory effect on them. And we concluded that the growth and development of primary roots are mainly controlled by *SiAAP9* expression levels, rather than long-term NO_3^- regulation.

***SiAAP9-OX* transgenic *Arabidopsis* lines exhibit tolerance to high concentrations of Glu and His.**

In order to clarify the amino acid substrates that *SiAAP9* may transport, we conducted an investigation on the tolerance of *SiAAP9* transgenic *Arabidopsis* plants to different types of amino acids. By creating various amino acid concentration gradients, we observed phenotypic differences when exposed to 10 mM Glu and 5 mM His (Fig. 6A-B), the results showed that *SiAAP9-OX* transgenic *Arabidopsis* lines had larger aboveground parts and longer roots compared to WT. While *SiAAP9-OX* transgenic *Arabidopsis* lines did not show significant tolerance to the other 17 amino acids (supplementary Fig. S6, S7). To eliminate the interference of inorganic nitrogen, we investigated the tolerance of *SiAAP9-OX* transgenic *Arabidopsis* lines to amino acids in medium with amino acids as the sole nitrogen source, we discovered that both the WT and *SiAAP9-OX* transgenic *Arabidopsis* lines wilted on a medium with Glu as the sole nitrogen source (Fig. 6C, E, F). Conversely, on the medium with His as the sole nitrogen source, the root length and fresh weight of the *SiAAP9-OX* transgenic *Arabidopsis* lines significantly increased compared to WT (Fig. 6D, G, H). These results indicated that *SiAAP9-OX* transgenic *Arabidopsis* lines are intolerant to high concentrations of Glu after lacking inorganic nitrogen, while remaining tolerant to excessive His.

The two proteins encoded by *SiAAP9L* and *SiAAP9S* have different subcellular localization.

To detect the subcellular localization of *SiAAP9S* and *SiAAP9L* and the effect of nitrogen on subcellular localization, 4-day-old seedlings cultured on 7 mM NO_3^- or 0 mM NO_3^- medium were used to figure it out. We found that *LOX-1#* is only localized on the plasma membrane, other organelles are basically undetectable and can only be observed under specific conditions (Fig. 7A). Compared to *LOX-1#*, *SiAAP9* may localized on organelles in other lines (Fig. 7B-D). Furthermore, by analyzing the fluorescence intensity of 40 cells, we observed that cells exposed to 0 mM NO_3^- exhibited stronger fluorescence signals

compared to those exposed to 7 mM NO_3^- . To further explore the specific localization of SiAAP9L and SiAAP9S, we utilized FM4-64 dye to locate on different organelles. The staining results indicate that LOX-1# has a high degree of plasma membrane localization, while other lines also have partial colocalization on TGN and PVC in addition to plasma membrane localization. Based on this observation, we hypothesize that a higher expression level of *SiAAP9* may disrupt the protein sorting process. It is known that BFA sensitive transport pathways consist of the efflux pathway from the Golgi to the plasma membrane and the degradation pathway from the Golgi to the vacuole (Pimpl et al. 2003). Surprisingly, after BFA treatment, we found that SiAAP9L exhibits significant colocalization with BFA compartment, while SiAAP9S does not. This suggests that

sequence deletion weakens the sensitivity of SiAAP9 to BFA. We did not observe any significant effect of WM treatment that can cause the fusion of vacuolar precursors to become larger (Wang et al. 2009) (supplementary Fig. S8A-D). On the other hand, we speculate that SiAAP9 protein is detained on ER, and ER staining results indicate that both SiAAP9L and SiAAP9S are localized on ER (supplementary Fig. S8E). Overall, the high expression of *SiAAP9* alters the subcellular localization of its encoded protein, with differences in localization between long and short proteins.

SiAAP9 may promote Glu uptake in Jingu 21 protoplast.

In order to further validate the transport of Glu by SiAAP9, we conducted a protoplast amino acid uptake assay. Empty vector, *OE-SiAAP9L*, and *OE-SiAAP9S* transformed into protoplasts of Jingu 21 were cultured under dark conditions for 4 hours using FITC labeled Glu. The results showed that the fluorescence intensity of protoplasts transformed with *OE-SiAAP9* was significantly higher than that of protoplasts transformed empty vector (Fig. 8), indicating that SiAAP9 can transport more Glu into foxtail millet cells.

Discussion

Higher concentration of amino acids transported to seeds by SiAAP9 transporter may inhibit the growth and development of *Arabidopsis* shoots and roots.

Amino acid content is a key indicator of the nutritional quality of crop grains (Sheoran et al. 2022). In this study, the content of amino acids in *SiAAP9-OX* transgenic *Arabidopsis* seeds is significantly higher than in WT, which may contribute to the inhibition of *Arabidopsis* growth and seed size (supplementary Fig. S4). And there have been reports indicating that overexpression of *OsAAP3* increased the content of amino acids in rice grains but inhibited the growth of the second axillary bud and filled grain number per plant (Lu et al. 2018). Similarly, the overexpression of *OsAAP5* in transgenic rice led to a notable increase in the levels of Lys, Arg, Val and Ala in leaf sheaths and leaves. However, it also had an inhibitory effect on the growth of tiller buds, resulting in slower overall growth (Wang et al. 2019). These results are similar

to the results of this study. But the effects of OsAAP1 (Ji et al. 2020) and OsAAP4 (Fang et al. 2021) are opposite. Among the 20 amino acids measured, Glu content is the highest, which may also be the reason why *SiAAP9-OX* transgenic *Arabidopsis* lines exhibit tolerance to high concentrations of Glu (Fig. 6A). Besides the transformation of foxtail millet protoplasts has been further confirmed (Fig. 8). Consequently, we can conclude that SiAAP9 may serve as a specific transporter for Glu. As a signaling molecule in plants, glutamate plays an important role in regulating plant growth and resisting adversity (Liao et al. 2022), reflecting overexpression of *SiAAP9* may inhibit crop yield but may improve crop nutritional quality.

In the absence of inorganic nitrogen, *SiAAP9-OX* transgenic *Arabidopsis* lines were intolerant to high concentrations of Glu.

In this study, we found that *SiAAP9-OX* transgenic *Arabidopsis* lines had longer roots and greater fresh weight than WT on the medium containing inorganic nitrogen and Glu (Fig. 6A). On the medium with Glu as the only nitrogen source, *SiAAP9-OX* transgenic *Arabidopsis* lines and WT both appeared wilting phenomenon (Fig. 6C). Therefore, we speculate that there may be a certain crosstalk between inorganic nitrogen and organic nitrogen, which makes *SiAAP9-OX* transgenic *Arabidopsis* lines intolerant to high concentrations of Glu in the absence of inorganic nitrogen. Previous studies have investigated the regulation of nitrate and glutamate signaling pathways on the structure and growth of *Arabidopsis* primary roots. With the increase of exogenous L-Glu concentration, the growth of *Arabidopsis* primary roots is inhibited and the branching of lateral roots increases (Walch-Liu et al. 2006). Furthermore, it has been shown that NRT1.1 inhibits Glu's inhibitory effect on the primary root by sensing nitrate signals through the primary root tip, thereby readjusting the growth, development, and root structure of the primary root (Walch-Liu and Forde 2008). Based on this, we speculate that SiAAP9, as inorganic nitrogen and amino acid transporter in plants, may be able to sense nitrate signaling, thereby inhibiting the Glu signaling pathway and reducing the tolerance of *SiAAP9-OX* transgenic *Arabidopsis* lines to high concentrations of Glu, further enabling the transformation of *OE-SiAAP9* into Jingu 21 protoplasts to absorb more Glu (Fig. 8).

Two transcripts of *SiAAP9* perform identical functions but exhibit different subcellular localizations.

Alternative splicing is the process of generating different mRNA transcripts from a single gene, resulting in variations in function and subcellular localization of these transcripts (Carvalho et al. 2013; Chaudhary et al. 2019; Graveley 2001; Kriventseva et al. 2003). AS plays a crucial role in plant development and stress responses (Filichkin et al. 2015; Staiger and Brown 2013; Szakonyi and Duque 2018). Previous studies have demonstrated that the two transcripts of *TaNAK* have contrasting roles in regulating plant

flowering time and morphology, and they also exhibit distinct subcellular localizations (Wu et al. 2022). In *Arabidopsis*, *PIN7a* and *PIN7b* exhibit nearly identical expression patterns and subcellular localization (Kashkan et al. 2022). Although *ScMYBAS1-2* and *ScMYBAS1-3* have opposing roles in regulating crop yield, both proteins are localized in the nucleus (Fávero Peixoto-Junior et al. 2018). Under stressful conditions, AS can generate transcripts with similar functions but different regulatory properties. For example, overexpressing both the long and short transcripts of *ZmPP2C26* in rice significantly reduces drought tolerance and impairs photosynthetic parameters (Lu et al. 2022). In salt stress, the two AS transcripts of the ring-type E3 ligase *SRAS1* exhibit opposing responses (Zhou et al. 2021) and *OsbZIP58β* shows lower transcriptional activity compared to *OsbZIP58α* under high-temperature conditions (Xu et al. 2020a). Similarly, *TaGS3* produces five splice variants, and overexpression of *TaGS3.5* significantly increases grain weight, while overexpression of *TaGS3.1* has the opposite effect (Ren et al. 2021). The two transcripts of *CiFD* (*CiFDα* and *CiFDβ*) in citrus involved in drought and low temperature induced flowering exhibited similar expression patterns in different tissues. However, they differed in terms of subcellular localization and transcriptional activity (Ye et al. 2023), which is consistent with the findings of this experiment. In this study, we found that the two transcripts of *SiAAP9* showed consistent expression patterns in different tissues of Jingu 21, as well as in response to NO_3^- induction and NO_3^- starvation (Fig. 2). Both transcripts were found to inhibit plant root length, rosette leaf area and plant height during growth and development (Fig. 3), as well as root growth and development in nitrate response and nitrate starvation phenotypes (Fig. 5). Therefore, we conclude that two transcripts of *SiAAP9* have similar functions. However, it was observed that *SiAAP9S* was less sensitive to BFA compared to *SiAAP9L* (Fig. 8). Further research is needed to investigate the specific alternative splicing mechanisms of *SiAAP9L* and *SiAAP9S* transcripts.

Overexpression of *SiAAP9* may lead to ER stress, which is detrimental to plant growth and development.

In this study, LOX-1# exhibited normal plasma membrane localization (Fig. 7A), which is consistent with the results of AAP transporters localization on the plasma membrane and nuclear membrane previously found in *Arabidopsis*, rice, cucumber and legume, etc (Garneau et al. 2018; Okumoto et al. 2004; Taylor et al. 2015; Wang et al. 2019; Yao et al. 2023). However, LOX-2#, SOX-1# and SOX-2# also have colocalization on various organelles (Fig. 7B-D), reflecting that the high expression of *SiAAP9* leads to ectopic localization of the encoded protein. At the same time, we also found that *SiAAP9* is collocated with ER (Fig. 8). ER plays a crucial role in various cellular processes such as protein synthesis, folding, assembly, transport, and lipid metabolism (Chung et al. 2016; Pastor-Cantizano et al. 2020). When encountering issues with protein folding and modification processes in the ER, the ER stress response is triggered (Poór et al. 2019; Singh et al. 2021). Environmental factors such as low temperature (Guo et al. 2022), high temperature (Singh et al. 2021), drought (Wang et al. 2017) and salt stress (Ozgur et al. 2014) can induce ER stress, triggering plants to adapt and withstand these conditions. Overexpression of *TabZIP60s* in *Arabidopsis* exhibit an activation of ER stress-related gene expression, which enhances

ability to withstand heat stress (Geng et al. 2018). And Overexpression of *NAC062* activates the expression of UPR (unfolded protein response) downstream genes induced by ER stress, which helps to alleviate ER stress (Yang et al. 2014). The inhibition of *NAA50* has been discovered to impede plant growth and development, resulting in a reduction in plant photosynthetic capacity. This decrease in capacity induces ER stress and triggers the activation of multiple defense pathways (Neubauer and Innes 2020). Therefore, we speculate that ectopic colocalization of SiAAP9 and ER may cause ER stress, thereby inhibiting transgenic *Arabidopsis* plant growth.

Conclusions

In our study, we confirmed that overexpression of *SiAAP9* inhibited the growth of vegetative organs in transgenic *Arabidopsis* but increased the concentration of most amino acids in the seeds. The protein encoded by low expression level of *SiAAP9* is located in the normal plasma membrane, while the protein encoded by high expression level is located in various organelles. Moreover, ectopic localization of excess SiAAP9 protein on ER may also be one of the important reasons for inhibiting plants. The role of the alternative splicing mechanism of *SiAAP9* in plants and whether the colocalization of SiAAP9 and ER will result in ER Stress phenomenon and hinder the growth of transgenic *Arabidopsis* plants need further research.

Declarations

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Author contributions

XY and JW conceived the study and supervised the work. RM and ZL carried out the molecular and plant phenotyping experiments. XK and YZ analyzed the corresponding data. YW, YM and YW assisted in preparing experimental materials. RM, JW and XY wrote the manuscript. SD, XL, LG, XC, GY, JW and XY critically revised the article. JW and XY provided funding. All authors agreed with the submission of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

References

1. Carvalho RF, Feijão CV, Duque P (2013) On the physiological significance of alternative splicing events in higher plants. *Protoplasma* 250:639-650. <https://doi.org/10.1007/s00709-012-0448-9>
2. Chaudhary S, Jabre I, Reddy ASN, Staiger D, Syed NH (2019) Perspective on alternative splicing and proteome complexity in plants. *Trends Plant Sci* 24:496-506. <https://doi.org/10.1016/j.tplants.2019.02.006>
3. Chen J, Duan W, Ren X, Wang C, Pan Z, Diao X, Shen Q (2017) Effect of foxtail millet protein hydrolysates on lowering blood pressure in spontaneously hypertensive rats. *Eur J Nutr* 56:2129-2138. <https://doi.org/10.1007/s00394-016-1252-7>
4. Chen Z, Zhang Y, Zhang J, Fan B, Zhou Y, Cui X (2021) Expression of AtAAP gene family and endosperm-specific expression of AtAAP1 gene promotes amino acid absorption in *Arabidopsis thaliana* and maize. *Agronomy* 11:1668. <https://doi.org/10.3390/agronomy11081668>
5. Chung KP, Zeng Y, Jiang L (2016) COPII paralogs in plants: functional redundancy or diversity? *Trends Plant Sci* 21:758-769. <https://doi.org/10.1016/j.tplants.2016.05.010>
6. Dinkeloo K, Boyd S, Pilot G (2018) Update on amino acid transporter functions and on possible amino acid sensing mechanisms in plants. *Semin Cell Dev Biol* 74:105-113. <https://doi.org/10.1016/j.semcdb.2017.07.010>
7. Fang Z, Wu B, Ji Y (2021) The amino acid transporter OsAAP4 contributes to rice tillering and grain yield by regulating neutral amino acid allocation through two splicing variants. *Rice* 14:2. <https://doi.org/10.1186/s12284-020-00446-9>
8. Fávero Peixoto-Junior R, Mara de Andrade L, Dos Santos Brito M, Macedo Nobile P, Palma Boer Martins A, Domingues Carlin S, Vasconcelos Ribeiro R, de Souza Goldman MH, Nebó Carlos de Oliveira JF, Vargas de Oliveira Figueira A, Creste S (2018) Overexpression of ScMYBAS1 alternative splicing transcripts differentially impacts biomass accumulation and drought tolerance in rice transgenic plants. *PLoS One* 13:e0207534. <https://doi.org/10.1371/journal.pone.0207534>
9. Filichkin S, Priest HD, Megraw M, Mockler TC (2015) Alternative splicing in plants: directing traffic at the cross roads of adaptation and environmental stress. *Curr Opin Plant Biol* 24:125-135. <https://doi.org/10.1016/j.pbi.2015.02.008>

10. Fischer W N, André B, Rentsch D, Krolkiewicz S, Tegeder M, Breitzkreuz K, Frommer W B (1998) Amino acid transport in plants. Trends Plant Sci 3:188-195. [https://doi.org/10.1016/S1360-1385\(98\)01231-X](https://doi.org/10.1016/S1360-1385(98)01231-X)
11. Garneau MG, Tan Q, Tegeder M (2018) Function of pea amino acid permease AAP6 in nodule nitrogen metabolism and export, and plant nutrition. J Exp Bot 69:5205-5219. <https://doi.org/10.1093/jxb/ery289>
12. Geng X, Zang X, Li H, Liu Z, Zhao A, Liu J, Peng H, Yao Y, Hu Z, Ni Z, Sun Q, Xin M (2018) Unconventional splicing of wheat TabZIP60 confer sheat tolerance in transgenic *Arabidopsis*. Plant Sci 274:252-260. <https://doi.org/10.1016/j.plantsci.2018.05.029>
13. Grant JE, Ninan A, Cripps-Guazzone N, Shaw M, Song J, Pet Ík I, Novák OE, Tegeder M, Jameson PE (2021) Concurrent overexpression of amino acid permease AAP1(3a) and SUT1 sucrose transporter in pea resulted in increased seed number and changed cytokinin and protein levels. Funct Plant Biol 48:889-904. <https://doi.org/10.1071/FP21011>
14. Graveley BR (2001) Alternative splicing: increasing diversity in the proteomic world. Trends Genet 17:100-107. [https://doi.org/10.1016/s0168-9525\(00\)02176-4](https://doi.org/10.1016/s0168-9525(00)02176-4)
15. Gu J, Li Z, Mao Y, Struik PC, Zhang H, Liu L, Wang Z, Yang J (2018) Roles of nitrogen and cytokinin signals in root and shoot communications in maximizing of plant productivity and their agronomic applications. Plant Sci 274:320-331. <https://doi.org/10.1016/j.plantsci.2018.06.010>
16. Guo J, Nie J, Chen Z, Wang X, Hu H, Xu J, Lu J, Ma L, Ji H, Yuan J, Xu B (2022) Cold exposure-induced endoplasmic reticulum stress regulates autophagy through the SIRT2/FoxO1 signaling pathway. J Cell Physiol 237:3960-3970. <https://doi.org/10.1002/jcp.30856>
17. Hori K, Sun J (2022) Rice Grain Size and Quality. Rice 15:33. <https://doi.org/10.1186/s12284-022-00579-z>
18. Ji Y, Huang W, Wu B, Fang Z, Wang X (2020) The amino acid transporter AAP1 mediates growth and grain yield by regulating neutral amino acid uptake and reallocation in *Oryza sativa*. J Exp Bot 71:4763-4777. <https://doi.org/10.1093/jxb/eraa256>
19. Kashkan I, Hrtyan M, Retzer K, Humpolíčková J, Jayasree A, Filepová R, Vo-ndráková Z, Simon S, Rombaut D, Jacobs TB, Frilander MJ, Hejátko J,
20. Friml J, Petrášek J, Růžička K (2022) Mutually opposing activity of PIN7 splicing isoforms is required for auxin-mediated tropic responses in *Arabidopsis thaliana*. New Phytol 233:329-343. <https://doi.org/10.1111/nph.17792>
21. Koch W, Kwart M, Laubner M, Heineke D, Stransky H, Frommer WB, Tegeder M (2003) Reduced amino acid content in transgenic potato tubers due to antisense inhibition of the leaf H⁺/amino acid symporter StAAP1. Plant J 33:211-220. <https://doi.org/10.1046/j.1365-313x.2003.01618.x>
22. Kriventseva EV, Koch I, Apweiler R, Vingron M, Bork P, Gelfand MS, Sunyaev S (2003) Increase of functional diversity by alternative splicing. Trends Genet 19:124-128. [https://doi.org/10.1016/S0168-9525\(03\)00023-4](https://doi.org/10.1016/S0168-9525(03)00023-4)

23. Li P, Chen Y, Lu J, Zhang C, Liu Q, Li Q (2022) Genes and their molecular functions determining seed structure, components, and quality of rice. *Rice* 15:18. <https://doi.org/10.1186/s12284-022-00562-8>
24. Liao HS, Chung YH, Hsieh MH (2022) Glutamate: a multifunctional amino acid in plants. *Plant Sci* 318:111238. <https://doi.org/10.1016/j.plantsci.2022.111238>
25. Liu S, Wang D, Mei Y, Xia T, Xu W, Zhang Y, You X, Zhang X, Li L, Wang N (2020) Overexpression of *GmAAP6a* enhances tolerance to low nitrogen and improves seed nitrogen status by optimizing amino acid partitioning in soybean. *Plant Biotechnol J* 18:1749-1762. <https://doi.org/10.1111/pbi.13338>
26. Lu F, Li W, Peng Y, Cao Y, Qu J, Sun F, Yang Q, Lu Y, Zhang X, Zheng L, Fu F, Yu H (2022) ZmPP2C26 alternative splicing variants negatively regulate drought tolerance in maize. *Front Plant Sci* 13:851531. <https://doi.org/10.3389/fpls.2022.851531>
27. Lu K, Wu B, Wang J, Zhu W, Nie H, Qian J, Huang W, Fang Z (2018) Blocking amino acid transporter OsAAP3 improves grain yield by promoting outgrowth buds and increasing tiller number in rice. *Plant Biotechnol J* 16:1710-1722. <https://doi.org/10.1111/pbi.12907>
28. Ma H, Cao X, Shi S, Li S, Gao J, Ma Y, Zhao Q, Chen Q (2016) Genome-wide survey and expression analysis of the amino acid transporter superfamily in potato (*Solanum tuberosum* L.). *Plant Physiol Biochem* 107:164-177. <https://doi.org/10.1016/j.plaphy.2016.06.007>
29. Marmagne A, Masclaux-Daubresse C, Chardon F (2022) Modulation of plant nitrogen remobilization and postflowering nitrogen uptake under environmental stresses. *J Plant Physiol* 77:153781. <https://doi.org/10.1016/j.jplph.2022.153781>
30. Neubauer M, Innes RW (2020) Loss of the acetyltransferase NAA50 induces endoplasmic reticulum stress and immune responses and suppresses growth. *Plant Physiol* 183:1838-1854. <https://doi.org/10.1104/pp.20.00225>
31. Okumoto S, Koch W, Tegeder M, Fischer WN, Biehl A, Leister D, Stierhof YD, Frommer WB (2004) Root phloem-specific expression of the plasma membrane amino acid proton co-transporter AAP3. *J Exp Bot* 55:2155-2168. <https://doi.org/10.1093/jxb/erh233>
32. Ozgur R, Turkan I, Uzilday B, Sekmen AH (2014) Endoplasmic reticulum stress triggers ROS signalling, changes the redox state, and regulates the anti-oxidant defence of *Arabidopsis thaliana*. *J Exp Bot* 65:1377-1390. <https://doi.org/10.1093/jxb/eru034>
33. Pastor-Cantizano N, Ko DK, Angelos E, Pu Y, Brandizzi F (2020) Functional diversification of ER stress responses in *Arabidopsis*. *Trends Biochem Sci* 45:123-136. <https://doi.org/10.1016/j.tibs.2019.10.008>
34. Peng B, Kong H, Li Y, Wang L, Zhong M, Sun L, Gao G, Zhang Q, Luo L, Wang G, Xie W, Chen J, Yao W, Peng Y, Lei L, Lian X, Xiao J, Xu C, Li X, He Y (2014) OsAAP6 functions as an important regulator of grain protein content and nutritional quality in rice. *Nat Commun* 5:4847. <https://doi.org/10.1038/ncomms5847>
35. Perchlik M, Tegeder M (2018) Leaf amino acid supply affects photosynthetic and plant nitrogen use efficiency under nitrogen stress. *Plant Physiol* 178:174-188. <https://doi.org/10.1104/pp.18.00597>

36. Pimpl P, Hanton SL, Taylor JP, Pinto-daSilva LL, Denecke J (2003) The GTPase ARF1p controls the sequence-specific vacuolar sorting route to the lytic vacuole. *Plant Cell* 15:1242-1256. <https://doi.org/10.1105/tpc.010140>
37. Poór P, Czékus Z, Tari I, Ördög A (2019) The multifaceted roles of plant hormone salicylic acid in endoplasmic reticulum stress and unfolded protein response. *Int J Mol Sci* 20:5842. <https://doi.org/10.3390/ijms20235842>
38. Ren X, Zhi L, Liu L, Meng D, Su Q, Batool A, Ji J, Song L, Zhang N, Guo L, Liu X, Li J, Zhang W (2021) Alternative splicing of TaGS3 differentially regulates grain weight and size in bread wheat. *Int J Mol Sci* 22:11692. <https://doi.org/10.3390/ijms222111692>
39. Sachdev N, Goomer S, Singh LR (2021) Foxtail millet: a potential crop to meet future demand scenario for alternative sustainable protein. *J Sci Food Agric* 101:831-842. <https://doi.org/10.1002/jsfa.10716>
40. Sanders A, Collier R, Trethewy A, Gould G, Sieker R, Tegeder M (2009) AAP1 regulates import of amino acids into developing *Arabidopsis* embryos. *Plant J* 59:540-552. <https://doi.org/10.1111/j.1365-313X.2009.03890.x>
41. Santiago JP, Tegeder M (2016) Connecting source with sink: the role of *Arabidopsis* AAP8 in phloem loading of amino acids. *Plant Physiol* 171:508-521. <https://doi.org/10.1104/pp.16.00244>
42. Schmidt R, Stransky H, Koch W (2007) The amino acid permease AAP8 is important for early seed development in *Arabidopsis thaliana*. *Planta* 226:805-813. <https://doi.org/10.1007/s00425-007-0527-x>
43. Shan Q, Wang Y, Li J, Gao C (2014) Genome editing in rice and wheat using the CRISPR/Cas system. *Nat Protoc* 9:2395-2410. <https://doi.org/10.1038/nprot.2014.157>
44. Sharma N, Niranjana K (2018) Foxtail millet: Properties, processing, health benefits, and uses. *Food Rev Int* 34:329-363. <https://doi.org/10.1080/87559129.2017.1290103>
45. Sheoran S, Kumar S, Ramtekey V, Kar P, Meena RS, Jangir CK (2022) Current status and potential of biofortification to enhance crop nutritional quality: an overview. *Sustainability* 14:3301. <https://doi.org/10.3390/su14063301>
46. Singh MB, Lohani N, Bhalla PL (2021) The role of endoplasmic reticulum stress response in pollen development and heat stress tolerance. *Front Plant Sci* 12:661062. <https://doi.org/10.3389/fpls.2021.661062>
47. Sonawala U, Dinkeloo K, Danna CH, McDowell JM, Pilot G (2018) Review: functional linkages between amino acid transporters and plant responses to pathogens. *Plant Sci* 277:79-88. <https://doi.org/10.1016/j.plantsci.2018.09.009>
48. Staiger D, Brown JW (2013) Alternative splicing at the intersection of biological timing, development, and stress responses. *Plant Cell* 25:3640-3656. <https://doi.org/10.1105/tpc.113.113803>
49. Szakonyi D, Duque P (2018) Alternative splicing as a regulator of early plant development. *Front Plant Sci* 9:1174. <https://doi.org/10.3389/fpls.2018.01174>

50. Taylor M R, Reinders A, Ward J M (2015) Transport Function of Rice Amino Acid Permeases (AAPs). *Plant Cell Physiol* 56: 1355-1363. <https://doi.org/10.1093/pcp/pcv053>
51. Tegeder M, Masclaux-Daubresse C (2018) Source and sink mechanisms of nitrogen transport and use. *New Phytol* 217:35-53. <https://doi.org/10.1111/nph.14876>
52. Tian R, Yang Y, Chen M (2019) Genome-wide identification and evolutionary and expression analysis of the wheat amino acid transporter, an important gene family involved in abiotic stresses. *ResearchGate* 2:66. <https://doi.org/10.21203/rs.2.19266/v1>
53. Tian R, Yang Y, Chen M (2020) Genome-wide survey of the amino acid transporter gene family in wheat (*Triticum aestivum* L.): Identification, expression analysis and response to abiotic stress. *Int J Biol Macromol* 162:1372-1387. <https://doi.org/10.1016/j.ijbiomac.2020.07.302>
54. Walch-Liu P, Liu LH, Remans T, Tester M, Forde BG (2006) Evidence that L-glutamate can act as an exogenous signal to modulate root growth and branching in *Arabidopsis thaliana*. *Plant Cell Physiol* 47:1045-1057. <https://doi.org/10.1093/pcp/pcj075>
55. Walch-Liu P, Forde BG (2008) Nitrate signalling mediated by the NRT1.1 nitrate transporter antagonises L-glutamate-induced changes in root architecture. *Plant J* 54:820-828. <https://doi.org/10.1111/j.1365-313X.2008.03443.x>
56. Wang B, Du H, Zhang Z, Xu W, Deng X (2017) BhbZIP60 from resurrection plant boea hygrometrica is an mRNA splicing-activated endoplasmic reticulum stress regulator involved in drought tolerance. *Front Plant Sci* 8:245. <https://doi.org/10.3389/fpls.2017.00245>
57. Wang H, Shen Q, Zhang F, Fu Y, Zhu Y, Zhao L, Wang C, Zhao Q (2023)
58. Heat-treated foxtail millet protein delayed the development of pre-diabetes to diabetes in mice by altering gut microbiota and metabolomic profiles. *Food Funct* 14:4866-4880. <https://doi.org/10.1039/d3fo00294b>
59. Wang J, Cai Y, Miao Y, Lam SK, Jiang L (2009) Wortmannin induces homotypic fusion of plant prevacuolar compartments. *J Exp Bot* 60:3075-3083. <https://doi.org/10.1093/jxb/erp136>
60. Wang J, Wu B, Lu K, Wei Q, Qian J, Chen Y, Fang Z (2019) The Amino acid permease 5 (OsAAP5) regulates tiller number and grain yield in rice. *Plant Physiol* 180:1031-1045. <https://doi.org/10.1104/pp.19.00034>
61. Wang S, Yang Y, Guo M, Zhong C, Yan C, Sun S (2020) Targeted mutagenesis of amino acid transporter genes for rice quality improvement using the CRISPR/Cas9 system. *Crop J* 8:457-464. <https://doi.org/10.1016/j.cj.2020.02.005>
62. Wang T, Li Y, Huang Y, Zhao X, Dong Z, Jin W, Huang W (2022) Amino acid permease 6 regulates grain protein content in maize. *Crop J* 10:1536-1544. <https://doi.org/10.1016/j.cj.2022.03.007>
63. Wu B, Zhang X, Hu K, Zheng H, Zhang S, Liu X, Ma M, Zhao H (2022) Two alternative splicing variants of a wheat gene TaNAK1, TaNAK1.1 and TaNAK1.2, differentially regulate flowering time and plant architecture leading to differences in seed yield of transgenic *Arabidopsis*. *Front Plant Sci* 13:1014176. <https://doi.org/10.3389/fpls.2022.1014176>

64. Wu M, Wu S, Chen Z, Dong Q, Yan H, Xiang Y (2015) Genome-wide survey and expression analysis of the amino acid transporter gene family in poplar. *Tree Genet Genomes* 11:1-20. <https://doi.org/10.1007/s11295-015-0908-4>
65. Xu H, Li X, Zhang H, Wang L, Zhu Z, Gao J, Li C, Zhu Y (2020a) High temperature inhibits the accumulation of storage materials by inducing alternative splicing of OsbZIP58 during filling stage in rice. *Plant Cell Environ* 43:1879-1896. <https://doi.org/10.1111/pce.13779>
66. Xu J, Kong F, Zhang H, Zhang W, Wu H, Chen G (2020b) First report of root rot caused by *Fusarium nygamai* on foxtail millet (*Setaria italica*) in China. *Plant Dis* 382:727-733. <https://doi.org/10.1094/PDIS-06-22-1388-PDN>
67. Yan J, Yang L, Liu Y, Zhao Y, Han T, Miao X, Zhang A (2022) Calcineurin B-like protein 5 (SiCBL5) in *Setaria italica* enhances salt tolerance by regulating Na⁺ homeostasis. *Crop J* 10:234-242. <https://doi.org/10.1016/j.cj.2021.06.006>
68. Yang G, Wei Q, Huang H, Xia J (2020) Amino acid transporters in plant cells: a brief review. *Plants* 9:967. <https://doi.org/10.3390/plants9080967>
69. Yang X, Yang G, Wei X, Huang W, Fang Z (2023) OsAAP15, an amino acid transporter in response to nitrogen concentration, mediates panicle branching and grain yield in rice. *Plant Sci* 330:111640. <https://doi.org/10.1016/j.plantsci.2023.111640>
70. Yang Y, Chai Y, Liu J, Zheng J, Zhao Z, Amo A, Cui C, Lu Q, Chen L, HuYG (2021) Amino acid transporter (AAT) gene family in foxtail millet (*Setaria italica* L.): widespread family expansion, functional differentiation, roles in quality formation and response to abiotic stresses. *BMC Genomics* 22:519. <https://doi.org/10.1186/s12864-021-07779-9>
71. Yang Z, Lu S, Wang M, Bi D, Sun L, Zhou S, Song Z, Liu J (2014) A plasma membrane-tethered transcription factor, NAC062/ANAC062/NTL6, mediates the unfolded protein response in *Arabidopsis*. *Plant J* 79:1033-1043. <https://doi.org/10.1111/tpj.12604>
72. Yao X, Li H, Nie J, Liu H, Guo Y, Lv L, Yang Z, Sui X (2023) Disruption of the amino acid transporter CsAAP2 inhibits auxin-mediated root development in cucumber. *New Phytol* 239:639-659. <https://doi.org/10.1111/nph.18947>
73. Yao X, Nie J, Bai R, Sui X (2020) Amino acid transporters in plants: identification and function. *Plants* 9:972. <https://doi.org/10.3390/plants9080972>
74. Ye L, Wu Y, Zhang J, Zhang J, Zhou H, Zeng R, Zheng W, Qiu M, Zhou J, Xie Z, Hu C, Zhang J (2023) A bZIP transcription factor (CiFD) regulates drought and low-temperature-induced flowering by alternative splicing in citrus. *J Integr Plant Biol* 65:674-691. <https://doi.org/10.1111/jipb.13390>
75. Zhang L, Garneau MG, Majumdar R, Grant J, Tegeder M (2015) Improvement of pea biomass and seed productivity by simultaneous increase of phloem and embryo loading with amino acids. *Plant J* 81:134-146. <https://doi.org/10.1111/tpj.12716>
76. Zhou Y, Li X, Guo Q, Liu P, Li Y, Wu C, Yang G, Huang J, Zhang S, Zheng C, Yan K (2021) Salt responsive alternative splicing of a RING finger E3 ligase modulates the salt stress tolerance by fine-

A 2000 bp
1000 bp

B 2000 bp
1000 bp
a b

C SiAAP9L Exon+Intron
SiAAP9S Exon+Intron

↓ Cut out this sequence on Exon4

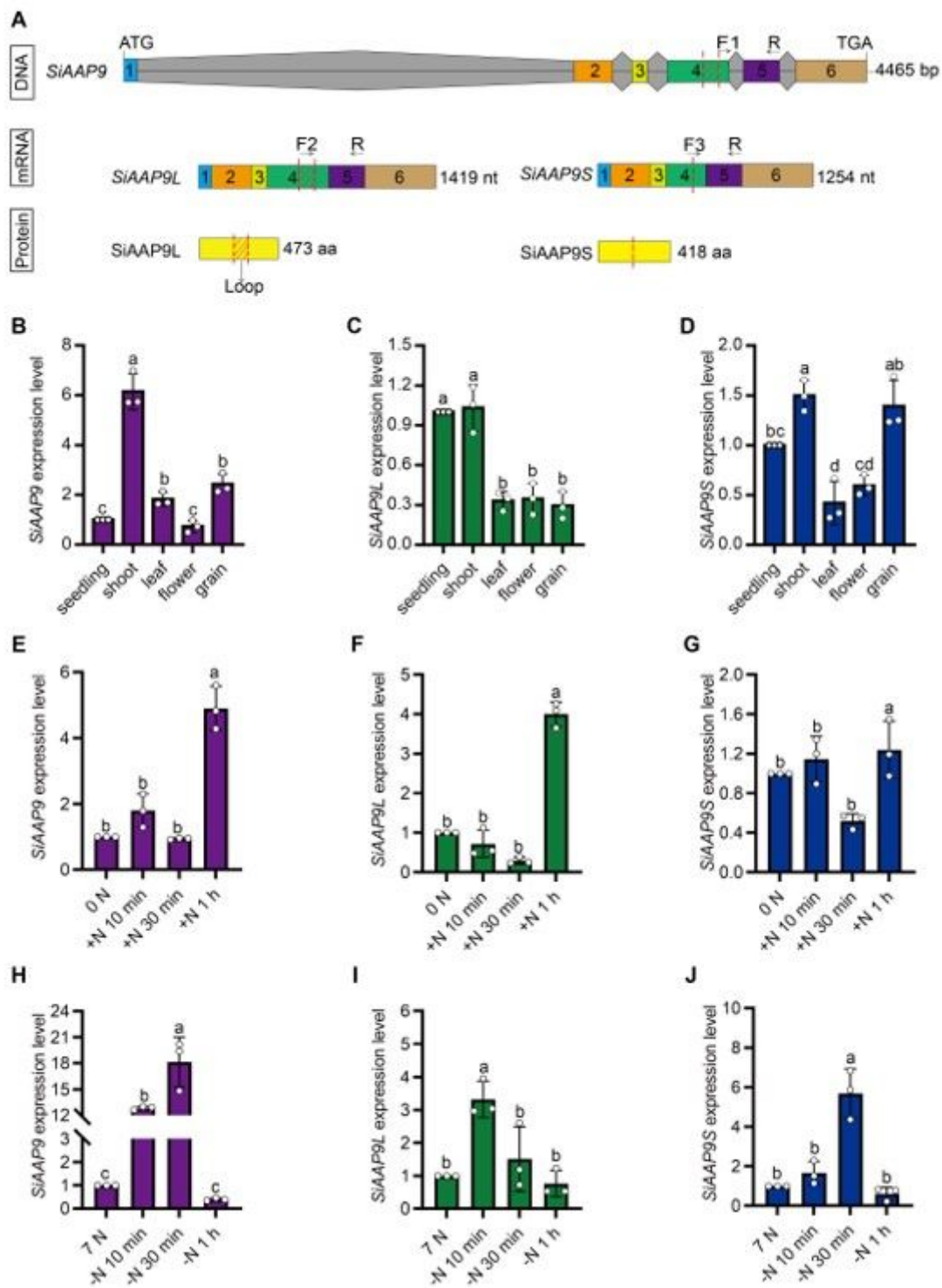


Figure 2

Analysis of *SiAAP9* expression patterns.

A The schematic diagram illustrates the specific primers design for distinguishing between *SiAAP9*, *SiAAP9L* and *SiAAP9S* transcripts.

B-D The expression levels of *SiAAP9*, *SiAAP9L* and *SiAAP9S* transcripts in different tissues of Jingdu 21.

E-G The expression levels of *SiAAP9*, *SiAAP9L* and *SiAAP9S* transcripts after adding NO_3^- for 10 min, 30 min, and 1 hour, respectively.

H-J The expression levels of *SiAAP9*, *SiAAP9L* and *SiAAP9S* transcripts after reducing NO_3^- for 10 min, 30 min, and 1 hour, respectively.

B-J Significant difference is analyzed based on one-way ANOVA by Duncan test, lowercase letters at $p < 0.05$.

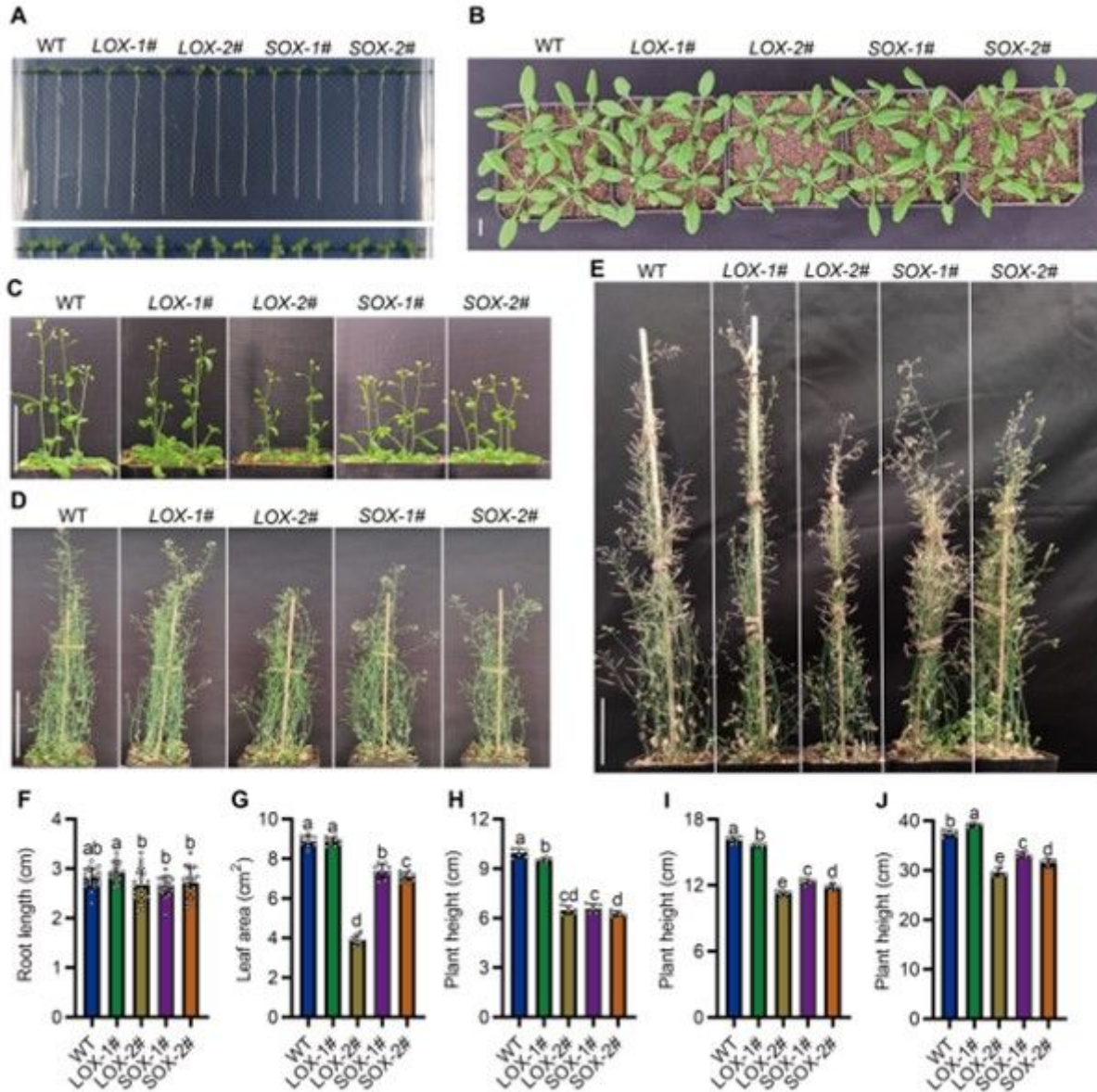


Figure 3

The growth phenotype analysis of WT and *SiAAP9-OX* transgenic *Arabidopsis* lines under normal conditions.

A Morphology of the seedlings at 7 days in WT and *SiAAP9-OX* transgenic *Arabidopsis* lines. Scale bar = 1 cm.

B Morphology of the rosette leaves at 3 weeks in WT and *SiAAP9-OX* transgenic *Arabidopsis* lines. Scale bar = 1 cm.

C-E Whole plant morphology of WT and *SiAAP9-OX* transgenic *Arabidopsis* lines at 4, 6 and 8 weeks. Scale bar = 5 cm.

F Primary roots length at 7 days was measured in WT and *SiAAP9-OX* transgenic *Arabidopsis* lines. Data represent means $\pm$ SD (n = 20).

G Rosette leaf areas at 3 weeks were measured in WT and *SiAAP9-OX* transgenic *Arabidopsis* lines. Data represent means $\pm$ SD (n = 10).

H-J Plant height at 4, 6 and 8 weeks were measured in WT and *SiAAP9-OX* transgenic *Arabidopsis* lines. Data represent means $\pm$ SD (n = 6).

F-J Significant difference is analyzed based on one-way ANOVA by Duncan test, lowercase letters at $p < 0.05$.

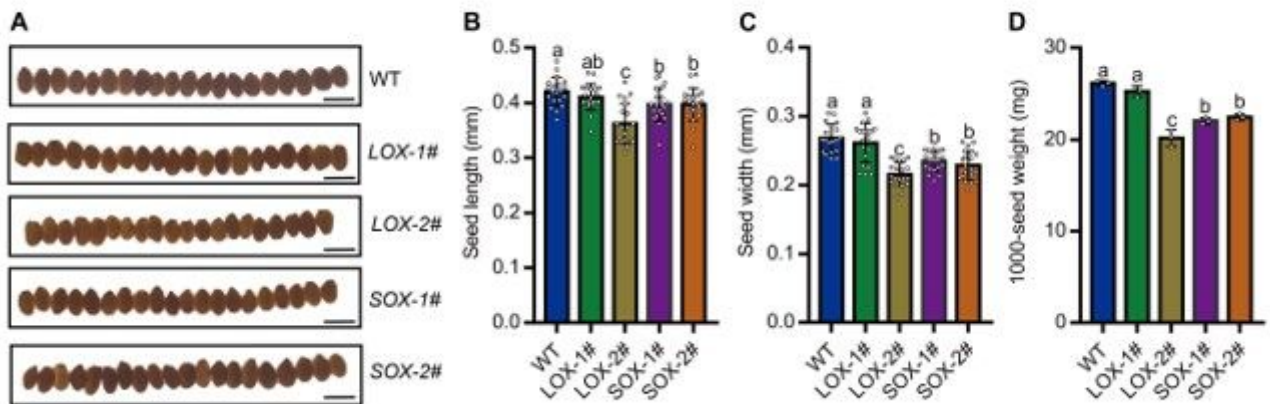


Figure 4

SiAAP9 inhibited the seed size in *SiAAP9-OX* transgenic *Arabidopsis* lines .

A Seed phenotypes of WT and *SiAAP9-OX* transgenic *Arabidopsis* lines after harvest. Scale bar = 500 μ m.

B-D Comparison of the length (**B**), width (**C**) and 1000-seed weight (**D**) of seeds in WT and *SiAAP9-OX* transgenic *Arabidopsis* lines. Data represent means $\pm$ SD (n = 20 for seed size and n = 3 for seed weight). Significant difference is analyzed based on one-way ANOVA by Duncan test, lowercase letters at $p < 0.05$.

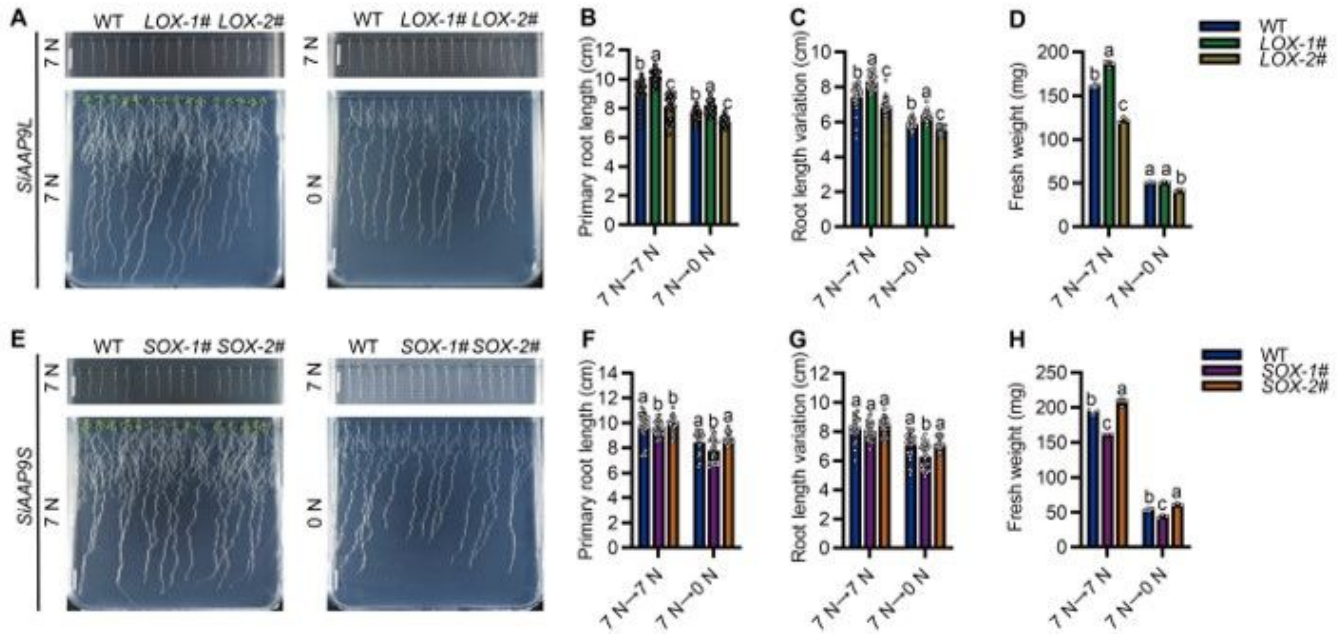


Figure 5

The phenotype analysis of WT and *SiAAP9-OX* transgenic *Arabidopsis* lines under NO_3^- free stress.

A, E WT, LOX-1#, LOX-2#, SOX-1# and SOX-2# were vertically cultivated on 7 mM NO_3^- medium for 4 days (upper panels), then were transferred to 0 mM NO_3^- medium and allowed to grow for an additional 8 days (lower panels). Scale bar = 1 cm.

B, F Primary root length was measured in WT and *SiAAP9-OX* transgenic *Arabidopsis* lines at 12 days. Data represent the mean $\pm$ SD (n = 20).

C, G Primary root length was measured in WT and *SiAAP9-OX* transgenic *Arabidopsis* lines after transferring the plate at 8 days. Data represent the mean $\pm$ SD (n = 20).

D, H Fresh weight was measured in WT and *SiAAP9-OX* transgenic *Arabidopsis* lines at 12 days. Data represent the mean $\pm$ SD (n = 20).

B-D, F-H Significant difference is analyzed based on one-way ANOVA by Duncan test, lowercase letters at $p < 0.05$.

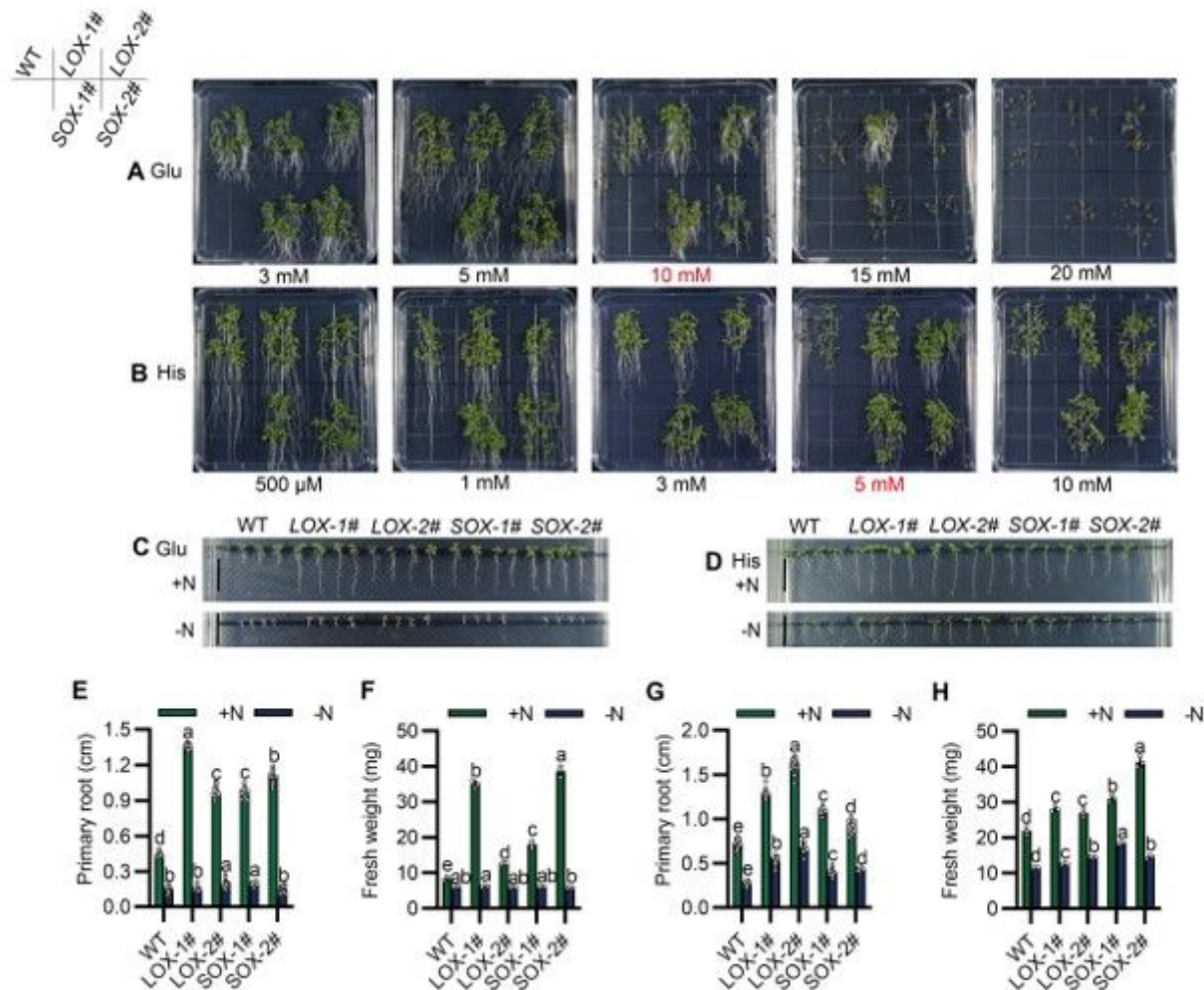


Figure 6

Overexpression of *SiAAP9* in *Arabidopsis* exhibited tolerance to high concentrations of Glu and His.

A, B WT and four *SiAAP9*-OX transgenic *Arabidopsis* lines were grown for 12 days on 1/2 MS medium containing Glu or His.

C, D WT and four *SiAAP9*-OX transgenic *Arabidopsis* lines were grown for 12 days on 1/2 MS medium with either normal N (+N) or 10 mM Glu (-N) or 5 mM His (-N) as the sole N source.

E, G Primary root length were measured on 1/2 MS medium containing 10 mM Glu or 5 mM His and 10 mM Glu (-N) or 5 mM His (-N) as the sole nitrogen source. Data represent the mean $\pm$ SD (n = 20).

F, H Fresh weight were measured on 1/2 MS medium containing 10 mM Glu or 5 mM His and 10 mM Glu (-N) or 5 mM His (-N) as the sole nitrogen source. Data represent the mean $\pm$ SD (n = 3).

E-H Significant difference is analyzed based on one-way ANOVA by Duncan test, lowercase letters at $p < 0.05$.

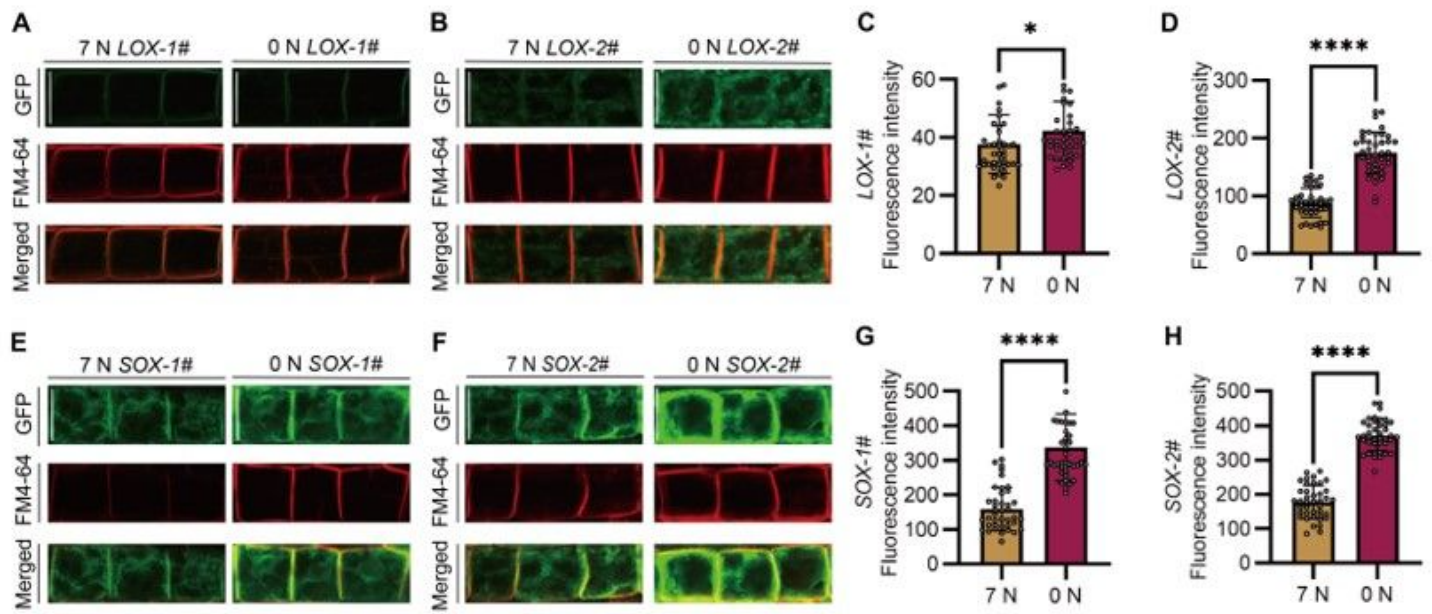


Figure 7

Subcellular localization analysis of SiAAP9L and SiAAP9S in *Arabidopsis* under two nitrate conditions.

A, B, E, F Four *SiAAP9-OX* transgenic *Arabidopsis* lines were cultured vertically on 1/2 MS medium for 4 days and meristematic zone cells of the roots were photographed using confocal laser scanning microscope. Scale bar = 20 μ m.

C, D, G, H Fluorescence intensity of GFP signal of four *SiAAP9-OX* transgenic *Arabidopsis* lines was calculated under 7 mM nitrate and 0 mM nitrate treatment, each treatment was calculated 40 cells, respectively. Data represent the mean $\pm$ SD ($n = 40$). Asterisks indicate significant differences based on one-way ANOVA followed by Student's t test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

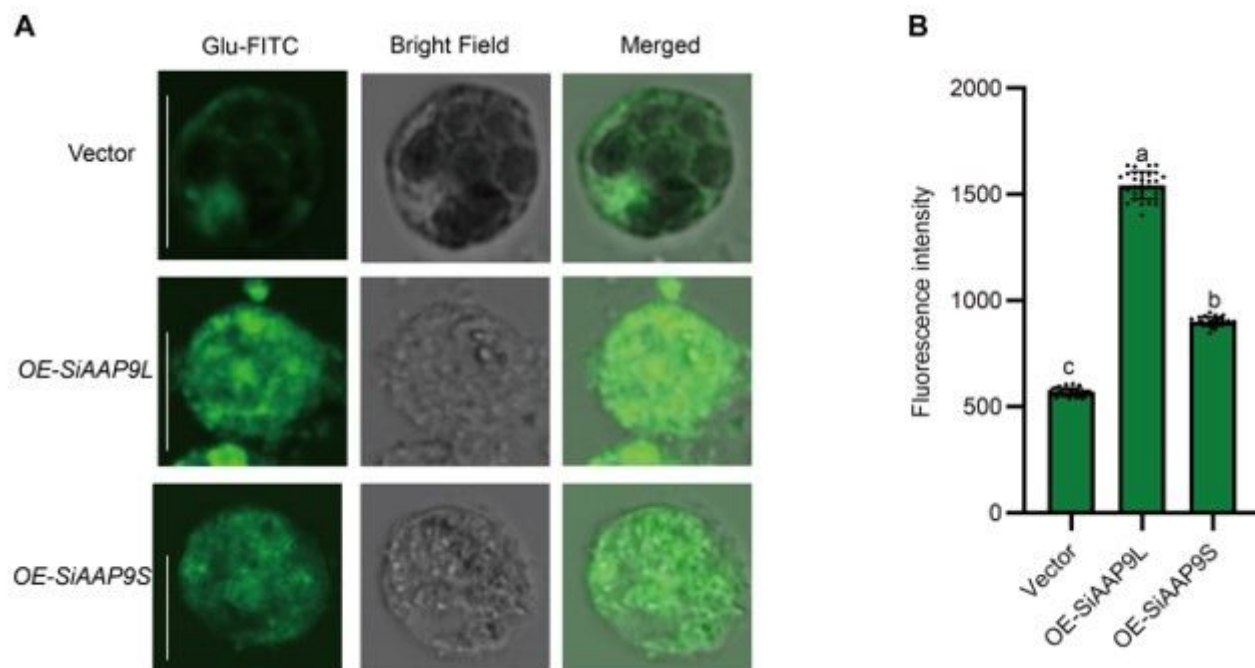


Figure 8

Amino acid uptake analysis in Jingu 21 protoplasts.

A Green fluorescence images of protoplasts transformed by empty vector, *OE-SiAAP9L* and *OE-SiAAP9S* under Glu-FITC treatment. Scale bars = 10 μ m.

B Statistical analysis of cell fluorescence signal intensity. Fluorescence intensity of 30 cells was measured when maintaining consistent setting parameters. Data represent the mean $\pm$ SD. Significant difference is analyzed based on one-way ANOVA by Duncan test, lowercase letters at $p < 0.05$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTable1.xlsx](#)
- [supplementaryfigures.docx](#)