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REGULAR ARTICLE

Molecular characterization of Gm*STOP1* homologs in soybean under Al and proton stress

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Abstract
Background and aims The Sensitive to Proton Rhizotoxicity1 (STOP1) transcription factor has been implicated in the regulation of aluminium (Al) stress and proton toxicity for several plant species. This study aimed to characterize STOP1 homologs in soybean.
Method Five *GmSTOP1* homologs were studied by transcriptional expression, subcellular localization and overexpression experiments.
Results Five GmSTOP1 homologs were nuclear-localized and exhibited transactivation activity. They constitutively expressed throughout the whole soybean plant. Their expressions were increased from 2 h, peaked at 4 h, returned to basal levels for the remaining duration of Al treatment but varied in aptitude and genotype. They were sensitive to pH conditions with various responses. Overexpression of *GmSTOP1a* in soybean hairy root increased the expression of the malate transporter gene *GmALMT1*, and decreased Al accumulation under Al stress. Its overexpression also regulated some pH-sensitive genes, including *GmSTOP1c* and *GmCIPK23*. Overexpression of *GmSTOP1a* in Arabidopsis slightly increase its Al resistance, and partially restored the root growth of the *atstop1* mutant under Al stress.
Conclusion *GmSTOP1a* contributes to both proton and Al resistance and plays a role similar to that of *AtSTOP1*. The functions of other four *GmSTOP1* genes need further clarified.

Keywords Aluminum toxicity · Soybean · Cys2His2 zinc finger protein · Transcriptional regulation · Proton resistance

Abbreviations

Al	Aluminum
CaMV	Cauliflower mosaic virus
STOP1	Sensitive to Proton Rhizotoxicity1
ART1	Aluminum resistance transcription factor 1

Introduction

The acceleration in the acidification of soils and waters is a global problem (Pannatier et al. 2005). Under natural conditions, acidification can be caused by the lesion of cations in soils, which can be promoted by improper crop-cultivating methods (Kochain et al. 2005). Through two nationwide surveys and paired comparisons in numerous individual sites, Guo et al. (2010) found that the soil pH declined from the 1980s to the 2000s in major Chinese crop-production areas, and the acidification process was primarily associated with nitrogen cycling

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release and base cation uptake. The major limitation to crop growth in acid soils is aluminum (Al) toxicity, accompanied by other acidic-related stresses, such as proton toxicity, Mn toxicity or phosphorus deficiency (Kochain et al. 2005). The identification of key genes regulating Al and acidic stress-related resistance will provide an effective strategy to improve crop adaptation to the acidic soil environment (Iuchi et al. 2008). For example, soybean root malate exudation is coordinately regulated by low pH, Al and P through *GmALMT1*, which might be the critical mechanism for soybean adaptation to acid soils (Liang et al. 2013).

Mutant *atstop1* was hypersensitive to both H^+ rhizotoxicity and Al^{3+} rhizotoxicity (Iuchi et al. 2007). Sensitive to Proton Rhizotoxicity1 (STOP1) regulates a range of genes involved in various functions, including Al resistance, ion homeostasis and pH-regulating metabolism (Sawaki et al. 2009). The unique homolog of *AtSTOP1* in Arabidopsis, *AtSTOP2,* can partially recover the gene transcript levels repressed by the *atstop1*-mutation and was suggested as a physiologically minor isoform of *AtSTOP1* (Kobayashi et al. 2014). *STOP1*-like proteins have been identified in some plant species, such as *Nicotiana tabacum*, *Lotus japonicus*, *Populus nigra* (black poplar), *Camellia sinensis* (Tea), *Physcomitrella patens* (moss), *Eucalyptus*, *Triticum aestivum* L (Wheat), and *Vigna umbellata* (rice bean) (Ohyama et al. 2013; Sawaki et al. 2014; Fan et al. 2015). Functional analyses, including in planta complementation assays, have revealed that STOP1-like proteins have varied functions within plant species. *Eucalyptus* STOP1-like protein complemented proton tolerance in an *atstop1* mutant and regulated the citrate-transporting *MATE* protein and an ortholog of *ALS3* (Sawaki et al. 2014). The complementation expression of *VuSTOP1* in rice bean, whose transcriptional expression was induced by both Al^{3+} and H^+ stress, significantly restored the H^+, but not the Al^{3+}, hypersensitivity of the *atstop1* mutant (Fan et al. 2015). Three homologous *STOP1* genes (*TaSTOP1-A*, *TaSTOP1-B*, and *TaSTOP1-D*) in wheat showed increased transcription levels under Al or proton toxicity (Garcia-Oliveira et al. 2013). However, the functions of these genes have not been studied.

ART1 (Aluminum resistance transcription factor 1) in rice has homologous Cis-2-His-2 zinc-finger domains but belongs to a different branch than STOP1 (Yamaji et al. 2009). OsART1 regulates multiple genes involved in Al detoxification at various cellular sites but does not regulate H^+ tolerance genes (Yamaji et al. 2009;; Chen et al. 2012; Xia et al. 2013). Closer homologies within the *ART1* branch (putative *ART1* orthologs) have been identified in maize (*Zea mays*) and rye (*Secale cereale*), suggesting that *ART1* is a transcription factor originally evolved in monocots (Yamaji et al. 2015).

Soybean microarray analysis revealed an increase of the *STOP1* transcript in soybean under Al stress (approximately 2-fold) (You et al. 2011). In the present study, five *STOP1* homologs blasted from the soybean genome were characterized based on their transcription expression, transactivation potential and subcellular localization. Functional analysis of *GmSTOP1a* was performed by overexpression in transgenic soybean hairy roots, Arabidopsis and *atstop1* mutant.

Material and methods

Plant cultivation and cDNA preparation

Soybean genotype Jiyu70 and Jiyu62 are considered Al-resistant and Al-sensitive genotypes, respectively, because the former showed higher relative root elongation and citrate efflux under Al stress compared with the latter in previous reports (Zhou et al. 2018). For hydroponic culture, soybean seeds of Jiyu 70 and Jiyu 62 were surface sterilized in 1.0% (*v*/v) sodium hypochlorite for 5 min, washed 3–4 times with tap water, and germinated in peat moss for 3 days at 25 °C in the dark. After germination, seedling of Jiyu 70 was transferred into nutrient solution for gene cloning and transcipitonal expression experiments. The cotyledons from Jiyu 62 were cut for hairy root inducing experients. The Jiyu 70 seedlings were cultured in 1-L plastic pots filled with aerated nutrient solution (Horst et al. 1992) (pH 4.5). The nutrient solution contained 750 μM KNO_3, 250 μM $Ca(NO_3)_2$, 325 μM $MgSO_4$, 10 μM KH_2PO_4, 20 μM Fe-EDTA, 8 μM H_3BO_3, and 0.2 μmol/L $(NH_4)_6Mo_7O_{24}$. The solution was renewed every other day. After culture for 7 days, the seedlings were transferred to 0.5 mM $CaCl_2$ solution overnight for the following treatments.

In the time course experiment, the seedlings were exposed to 30 μM $AlCl_3$ in 0.5 mM $CaCl_2$ solution, and 0–1 cm root apices were excised at 0, 2, 4, 8, 12, and 24 h. For other metal stresses, the seedlings were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) (control) or 0.5 mM $CaCl_2$ solutions (pH 4.5) containing 30 μM $AlCl_3$, 25 μM $CdCl_2$, 10 μM $LaCl_3$, or 0.5 μM $CuCl_2$. The 0–1 cm root apices were excised at 4 h stress exposure.

For the root localization experiments, the seedlings were exposed to 30 μM $AlCl_3$ in 0.5 mM $CaCl_2$ solution. The 0–1 cm root apices were excised at 0 h, and 0–1, 1–2, and 2–3 cm root segments were excised at 4 h after Al exposure. All hydroponic experiments were performed in a controlled growth chamber at 25 °C day: 22 °C night temperatures, 60% constant relative humidity, 14 h light:10 h dark cycles, and 300 μmol m^{-2} s^{-1} of light intensity during the day.

Jiyu70 was grown at the Agricultural Trial Station of Jilin University. The following basic characteristics of the top 0–20 cm soil layer were recorded: pH 6.5, 49.4 ± 4.8 g/kg available nitrogen, 11.8 ± 4.1 g/kg available P, 170 ± 6.2 g/kg available K and 21.8 ± 3.7 g/kg organic carbon. Eighty days after sowing, the soybean plants were harvested. Soybean pods, flowers, leaves, and shoots were separated. After careful washing, the intact soybean roots were also cut. The harvested tissues were collected by liquid nitrogen and stored at −80 °C until subsequent RNA extraction. After cultivation for eighty days, the roots, shoots, leaves, flowers and pods were sampled in the field-grown soybean. All the samples were instantly frozen in liquid nitrogen and stored at −80 °C until subsequent RNA isolation.

Total RNA was isolated using total RNA extraction kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. First–strand cDNA synthesis was performed with 2 μg of total RNA using reverse transcriptase kit (Thermo Scientific, Massachusetts, USA) .

Gene cloning and bioinformatics analysis of the *GmSTOP1* genes

The homologous gene sequences of *STOP1* were blasted from soybean genomes at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) with "soybean STOP1" as the key words. *GmSTOP1a* (*Glyma.10 g35940*), *GmSTOP1b* (*Glyma.12 g08680*), *GmSTOP1c* (*Glyma.12 g30285*), *GmSTOP1d* (*Glyma.18 g02010*) and *GmSTOP1e* (*Glyma.08 g14320*) were named accordingly. Specific primers were designed by Primer 5.0 software according to the sequence and/or considering the enzyme cutting locus of the applied vectors. The primer sequences are listed in Table 1S. The genes were amplified with the cDNA templatetranscribed from the RNA of Jiyu70 root apices treated with Al for 4 h.

The sequences *STOP1* or *ART1* genes in other plant species were blasted from NCBI or got from references. *Arabidopsis thaliana (AtSTOP1, At1g34370; AtSTOP2,At5g22890), Nicotiana tabacum (NtSTOP1,AB811781), Triticum aestivum (TaSTOP1-A,KF034801; TaSTOP1-B,KF034802; TaSTOP1-D, KF034795), Camellia sinensis (CsSTOP1,AB811780), Populus nigra (PnSTOP1,AB81178), Lasianthus japonicas (LjSTOP1,AB811782), Eucalyptus (EguSTOP1,AB826006), Vigna umbellate (VuSTOP1,KP637172), Oryza sativa (OsART1,Os12g0170400), Physcomitrella patens (PpSTOP1,AB811778), Sorghum bicolor (SbSTOP1,Sb07g023890; SbSTOP1–1,Sb04g023670; SbSTOP1–2,Sb07g023890; SbSTOP1–3,Sb03g041170), Vitis vinifera (VvSTOP1,AB811779).* Multiple sequence alignment was achieved through ClustalX and GeneDoc software (Pittsburgh Supercomputing Center, Pittsburgh, USA). The four zinc finger domains were indicated after InterproScan function domain analysis (http://www.Ebi.ac.uk/InterProScan/). The phylogenetic relationship with other *STOP1* or *ART1* genes was analyzed by generating a phylogenetic tree with MEGA 5.0.

Transcriptional expressions of five *GmSTOP1* genes

Quantitative real-time PCR (qRT-PCR) was performed to determine the transcriptional expression level of five *GmSTOP1* genes in soybean, with *β-Tubulin* (GenBank ID: 100811275) as an internal standard. The qRT-PCR analysis was conducted with an M × 3005P machine (Stratagene, La Jolla, CA,USA). The reaction system (25 μl) contained following contents: 2 μl of cDNA template (50–100 ng), 1 μl of 10 mM gene-specific primer mixture of forward primer and reverse primer, 12.5 μl of 2× SYBR Premix Ex Taq (TaKaRa, Bio Inc.), and 9.5 μl of double-distilled H_2O. The reaction was performed under the following conditions: 1 cycle for 30 s at 95 °C, 30 cycles for 5 s at 95 °C and 20 s at 60 °C, and 1 cycle for 60 s at 95 °C, 30 s at 55 °C, and 30 s at 95 °C for the melting curve analysis. The relative expression level of the genes was computed by the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen 2001).

Subcellular localization of five *GmSTOP1* proteins

The CDS of *STOP1* and homologous genes were amplified with forward and reverse primers and subsequently cloned into the pENSG-N-*GFP* vector with the

cauliflower mosaic virus (CaMV) 35S as a promoter. The resulting *35S::SbSTOP1::GFP* plasmid (and an additional *35S::GFP* plasmid) was introduced into Arabidopsis protoplasts. The resulting construct was fully sequenced to assess the sequence accuracy. The Green fluorescent protein (GFP) signal was observed via microscopy (Zeiss 2012 Observer A1, Göttingen, Germany).

Transactivation potentials assay

Full-length sequences of *GmSTOP1a*, *GmSTOP1b*, *GmSTOP1c*, *GmSTOP1d* and *GmSTOP1e* were respectively inserted into vector of pBridge. Each plasmid with *GmSTOP1s* was separately transformed into yeast strain Y190 carrying GAL1 promoter and HIS3 reporter gene (Clontech, PT3024–1). Yeast cell were cultured on SD/−Trp medium at 30 °C for 3 days. Picked three yeast colonies into 100ul ddH_2O, and dropped it on SD/−Trp-His medium at 30 °C for 2d to observe their growth.

Overexpression of *GmSTOP1a* in soybean hairy roots

The cloned Gm*STOP1a* was amplified using forward and reverse primers with a BamH1 restriction site and subsequently ligated between CaMV 35S and the luciferase tag of the pCAMBIA3301-actin-2*FLAG vector.

The resulting construct was sequenced and electroporated into strain K599. The transformation of soybean Jiyu62 was performed according to Subramanian et al. (2005), with some modifications. The transgenic hairy roots were selected by luciferase activity (the scanning value greater than 3000 was considered as successful transformation). The hairy roots induced by only K599 were considered as wild type (WT). For Al stress study, both transgenic and WT hairy roots were treated in 0.5 mM $CaCl_2$ solution (pH 4.5) including 0 or 30 μM $AlCl_3$ within a 5-ml plastic tube. Root apices (0–1 cm) were cut and stored at −80 °C for Al concentration assay and RNA isolation. The Al concentration of hairy root apices was extracted by 2 M HCl for 48 h, and detected by an atomic adsorption spectrophotometer equipped with a graphite furnace atomizer (PerkinElmer AAnalyst 700, USA). Transgenic and WT hairy roots were also cultured in 0.5 mM $CaCl_2$ solution with pH as 3.5, 4.5 or 5.5. After 4 h, root apices of 0–1 cm were excised for RNA isolation. The transcriptional expression of *GmALMT1* (*Glyma.03 g36060*), *GmSTOP1c* (*Glyma.12 g30285*), *GmCIPK23* (*Glyma.09 g11770*), *GmPIPG1* (*Glyma.19 g32700*), and *GmGDH1* (*Glyma.16 g04560*) were studied in the hairy roots under Al stress or different pH conditions.The sequences of *GmALMT1*, *GmSTOP1c*, *GmCIPK23*, *GmPIPG1*, and *GmGDH1* were blasted from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) according to their corresponding homologous genes reported in Arabidopsis (*AtALMT1, AtSTOP2, AtCIPK23*, *AtPIPG1*, and *AtGDH1*) (Sawaki et al. 2009).Their primers were designed by using Primer 5.0 online software and listed in Table S1.

Heterologous overexpression of *GmSTOP1*a in an Arabidopsis ecotype and *atstop1 mutant*

The mutant *atstop1* was acquired from SALK (114108). The constructs of pCAMBIA3301-actin-2*FLAG vector with the coding regions of *GmSTOP1* under the control of the CaMV 35S promoter was introduced into an *Agrobacterium tumefaciens* strain (Agl0) and subsequently transformed into Arabidopsis ecotype (Col-4) or the *atstop1* mutant by the floral dip method (Clough and Bent 1998). The transgenic seedlings were screened by spraying with Basta herbicide and confirmed by luciferase activity measurements. Luciferase activity value in Arabidopsis greater than 10,000 was considered to be a successful transformation. Homozygous T3 lines were cultured in Al-containing media for an Al sensitivity assay by measuring relative root elongation according to Sun et al. (2014). Two independent transgenic lines were sown in MS medium for 5 d, then transferred to solid agar medium supplied with 4.3 mM $CaCl_2$ and 3% sucrose containing 0 or 100 μM $AlCl_3$ (pH 4.5). Their root lengths were measured before and after 2 d of Al treatment. The relative root elongation (RRE) was computed as (root elongation in Al treatment/root elongation in –Al treatment) × 100. T3 lines were also cultured in different pH media for the proton sensitivity assays. The Arabidopsis seeds were sown in MS medium to grow for 5 d. Seedlings were transferred into solid agar medium supplied with 4.3 mM $CaCl_2$ and 3% sucrose with pH 4.2, 4.7 or 5.5. Their root lengths were measured before and after 2 d of different pH treatment in the solid medium. Images of the representative seedlings were obtained by digital camera (Nikon).

Statistical analysis

Each result in transcriptional expression and Al contents was the mean of at least three replicates. Each result in the

relative root elongation or root elongation of Arabidopsis represented the means of fifteen to twenty replicates. The significance of differences among treatments or transgenic lines were analysed by *t*-test methods using DPS 11.0 edition for windows (Tang and Zhang 2012).

Results

Bioinformatics analysis of five STOP1-like genes in soybean

Five *STOP1*-like genes were blasted from the soybean genome and named as *GmSTOP1a*, *GmSTOP1b*, *GmSTOP1c*, *GmSTOP1d*, and *GmSTOP1e*. InterproScan function domain analysis indicated that the corresponding proteins each included four zinc finger domains and belonged to the Cys2His2 zinc finger family proteins (Fig. 1b). The five soybean STOP1-like proteins had relatively conserved four zinc finger domains, with high variation at the N- and C-termini (Fig. 1b). Phylogenetic analysis indicated that GmSTOP1a clusters closely with VuSTOP1 (*Vigna umbellate*), AtSTOP1 (Arabidposis) and NtSTOP1 (*Nicotiana tabacum*). The other four STOP1-like proteins, including GmSTOP1b, GmSTOP1c, GmSTOP1d, and GmSTOP1e, clustered more closely with AtSTOP2. The STOP1-like protein sequence varied within dicots and monocot, and the five STOP1-like proteins in soybean grouped differently from OsART1 (*Oryza sativa*), TaSTOP1A, TaSTOP1B, and TaSTOP1D (*Triticum aestivum*) (Fig. 1a).

The transcriptional expression pattern of *GmSTOP1* genes

Five *GmSTOP1* genes were constitutively expressed in soybean root apices and exhibited similar transcriptional expression in the Al-treated time course experiment (Fig. 2). Their expression was maintained within 2.5 to 6.5-fold levels at 4 h following Al treatment (Fig. 2a, b, c, d and e). Much higher expression levels were observed at *GmSTOP1a* (6.5 fold) and *GmSTOP1b* (4.5 fold) at 4 h (Fig. 2a, b). To determine whether *GmSTOP1s* specifically responds to Al stress, we compared the effects of Al stress with those of other metals and different pH conditions. *GmSTOP1c* was inhibited by Cd^{2+} or La^{3+} stress (Fig. 3c). The transcriptional expression levels of *GmSTOP1b* and *GmSTOP1e* were also increased under Cu^{2+} stress (Fig. 3b, e). *GmSTOP1a*, *GmSTOP1b* and *GmSTOP1c* showed similar expression trends under different pH conditions (Fig. 4a, b and c). These genes displayed lower expression at pH 4.5 and increased expression at a low pH of 3.5 or higher pH of 5.5. This finding indicates that these genes are sensitive to pH regulation. The expression of *GmSTOP1d* showed higher expression at pH 5.5 (Fig. 4d). *GmSTOP1e* was insensitive to pH conditions and remained constant under the three different pH conditions (Fig. 4e).

The expression of the five *GmSTOP1* genes was increased by Al treatment in the soybean roots (0–3 cm) (Fig. 5 a, b, c,d and e). The Al-increased expression of *GmSTOP1a* and *GmSTOP1e* in 0–1 cm root apices was higher than that found in the more basal regions (1–2 cm and 2–3 cm) (Fig. 5 ae).

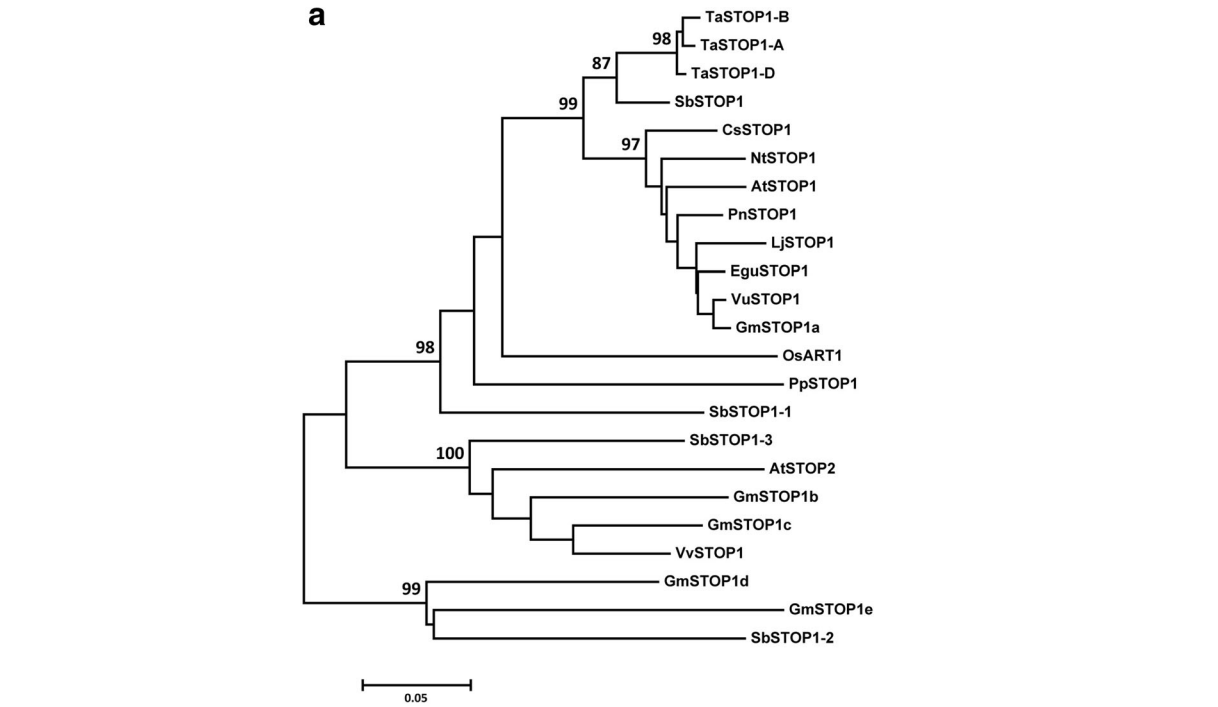
Five *GmSTOP1* genes were distributed throughout the roots, shoots, leaves, flowers and pods of soybean plants grown in the field under neutral soil conditions (Fig. 6a, b, c, d and e). Specifically, *GmSTOP1b*, *GmSTOP1c*, and *GmSTOP1e* had higher expression levels in the pod compared with those in the root (Fig. 6b,c and e).

Subcellular localization of five soybean STOP1 proteins

The subcellular localization of the five STOP1 proteins was examined by transiently expression assays with STOP1::GFP translational fusion in Arabidopsis protoplast cells. The fluorescence of each of the five of the STOP1::GFP fusion proteins was localized to the cell nucleus of the Arabidopsis protoplast. In contrast, the fluorescence of cells transformed with the only GFP vector was associated with the nucleus and cytosol. Thus, five GmSTOP1 proteins were localized to the nucleus (Fig. 7).

Transcription activities of five GmSTOP1 proteins

As the putative transcription factor of Cys2His2 (C2H2)-type zinc fingers family, each of the five GmSTOP1 proteins was fused to the pBridge vector to evaluate their transactivation potential by yeast transcriptional activation assays. The transformation of the pBridge vector carrying each of the five *GmSTOP1* genes could help the yeast to grow normally in Trp/His-deficient medium, exhibiting functions of self-activation. Thus, each of the five GmSTOP1proteins has transactivation activity (Fig. 8).


a
TaSTOP1-B
TaSTOP1-A
TaSTOP1-D
SbSTOP1
CsSTOP1
NtSTOP1
AtSTOP1
PnSTOP1
LjSTOP1
EguSTOP1
VuSTOP1
GmSTOP1a
OsART1
PpSTOP1
SbSTOP1-1
SbSTOP1-3
AtSTOP2
GmSTOP1b
GmSTOP1c
VvSTOP1
GmSTOP1d
GmSTOP1e
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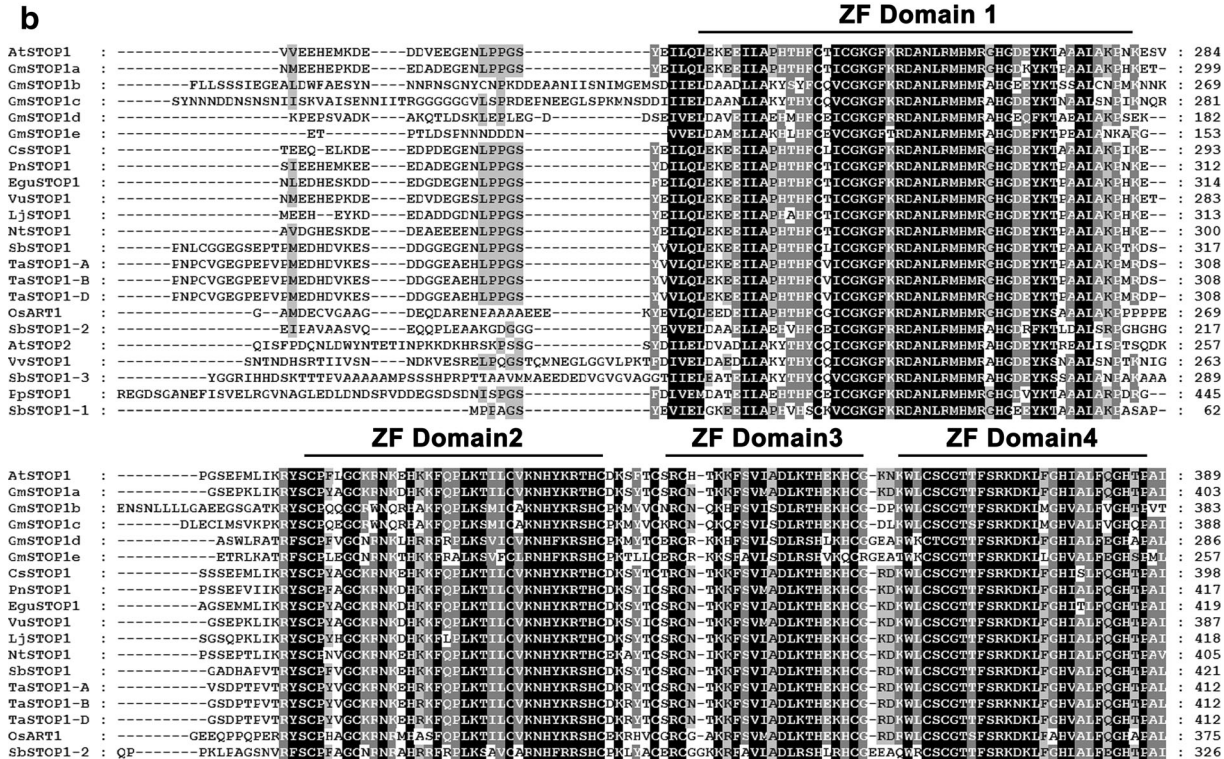
b
ZF Domain 1
ZF Domain2
ZF Domain3
ZF Domain4

◀ **Fig. 1 Phylogenetic tree and multiple sequence alignment of GmSTOP1 with known STOP1 orthologous proteins from other plant species.** Phylogenetic tree (**a**) and multiple sequence alignment (**b**) of the amino acid sequences of GmSTOP1 and orthologous proteins from other plant species, including *Arabidopsis thaliana* (AtSTOP1, At1g34370, AtSTOP2, and At5g22890), and *Oryza sativa* (OsART1 and Os12g0170400). Identical residues are shown on a black background, and conservative substitutions are shown on a gray background. Lines depict zinc-finger (ZF) domains as predicted in Arabidopsis by Englbrecht et al. (2004)

Overexpression of *GmSTOP1a* in soybean hairy roots

Overexpression of *GmSTOP1a* in soybean hairy roots resulted in the higher expression of *GmSTOP1a* (Fig. 9a) and *GmALMT1* (Fig. 9b) under either –Al or + Al conditions compared with that of K599-induced wild-type hairy roots (WT). Less Al concentration were also found within root apices of *GmSTOP1a*-OE compared with those of WT (Fig. 9c).

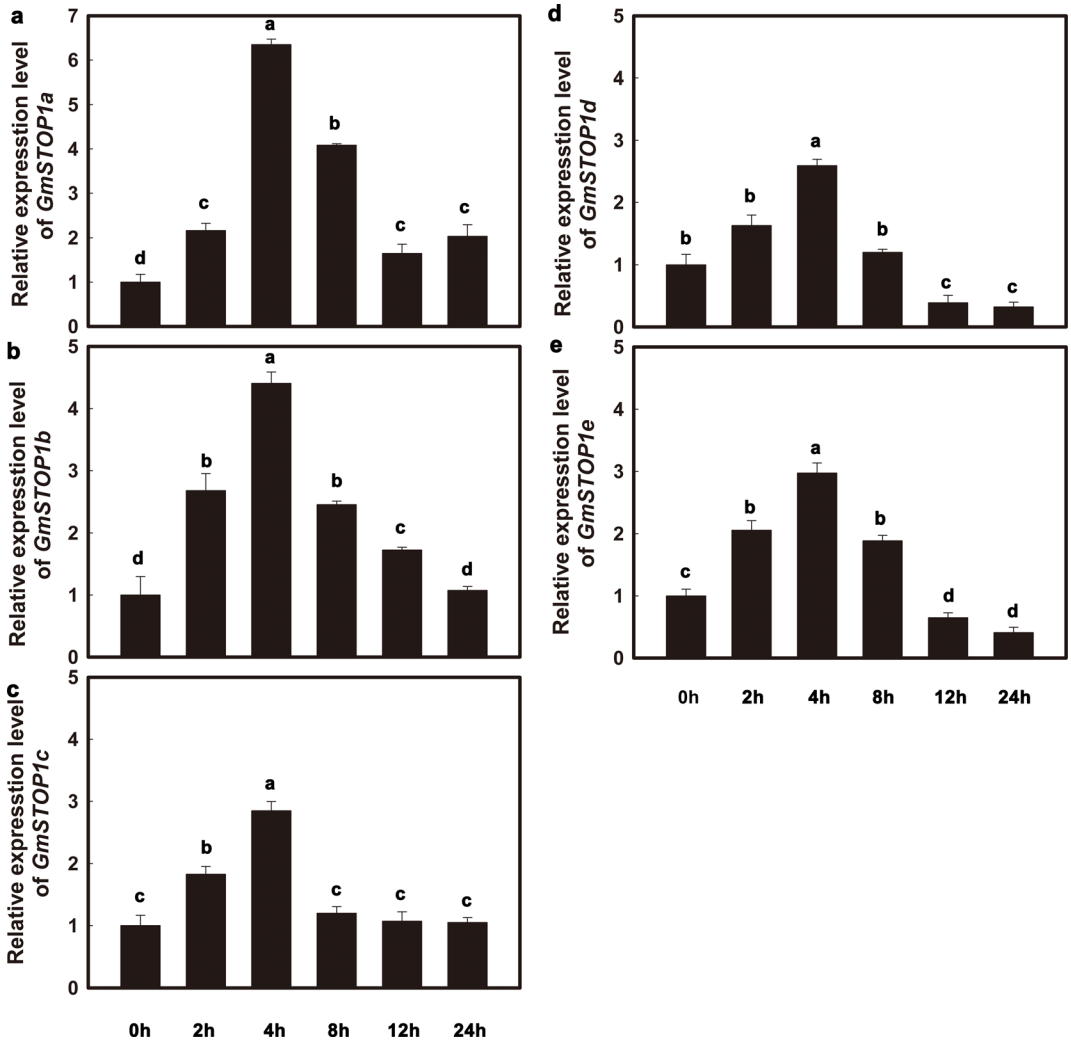

Fig. 2 Temporal expression of five *GmSTOP1* genes in soybean root apices under Al stress. Seven-day-old soybean seedlings were exposed to 0.5 mM $CaCl_2$ solution containing 30 μM $AlCl_3$ (pH 4.5). The 0–1 cm root apices were excised after 0, 2, 4, 8, 12, and 24 h Al treatment to study temporal expression. The expression levels of *GmSTOP1a* (**a**), *GmSTOP1b* (**b**), *GmSTOP1c* (**c**), *GmSTOP1d* (**d**) and *GmSTOP1e* (**e**) were examined by qRT-PCR, with *β-tubulin* as the reference gene. Data are represented as means ±standard deviation (SD) of three biological replicates. Different letters above column represented significantly different ($p < 0.05$, *t*- test)

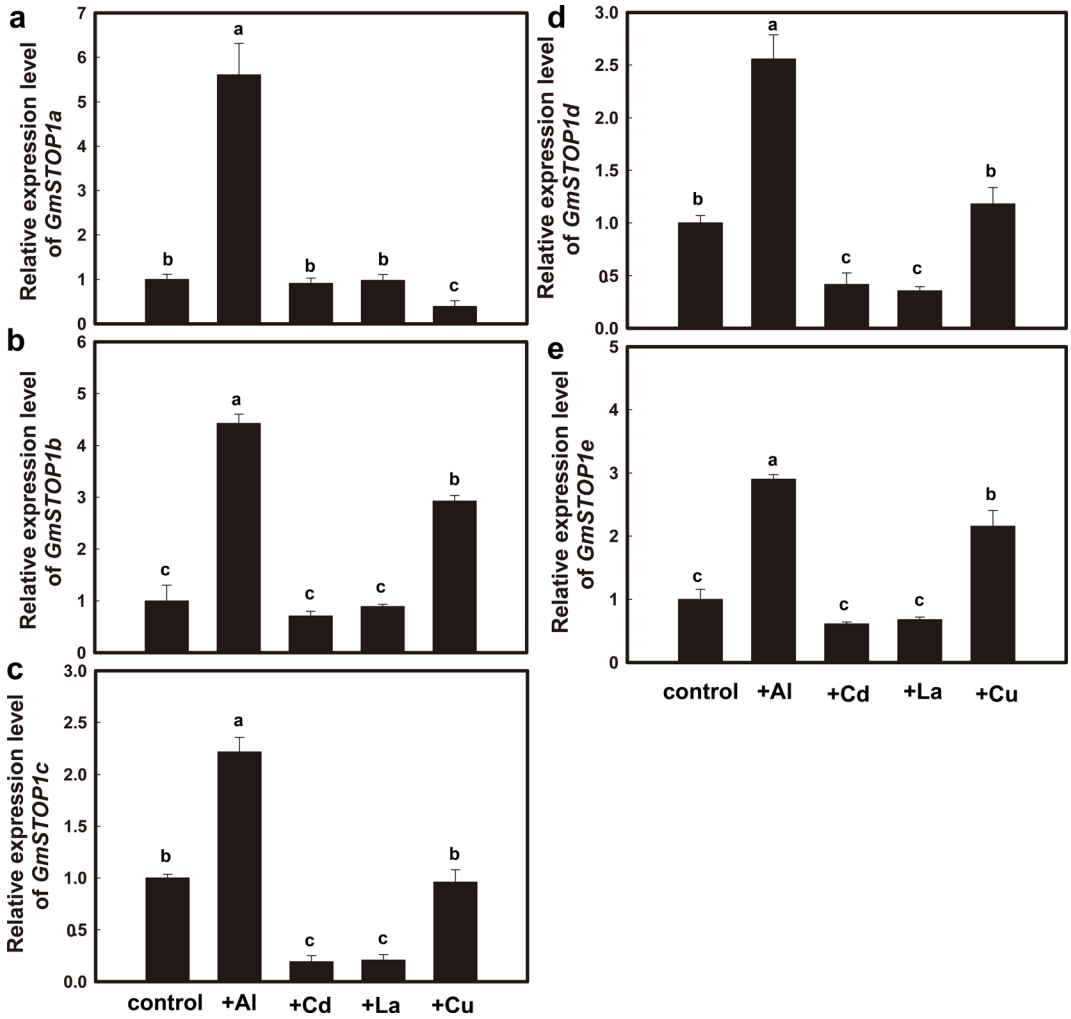

Fig. 3 Transcriptional expression of five *GmSTOP1* genes in response to Cd^{2+}, La^{3+}, Cu^{2+}, and Al^{3+} stress in soybean root apices. Transcriptional expression of *GmSTOP1a* (**a**), *GmSTOP1b* (**b**), *GmSTOP1c* (**c**), *GmSTOP1d* (**d**) and *GmSTOP1e* (**e**) under Cd^{2+}, La^{3+}, Cu^{2+} and Al^{3+} stresses in soybean root apices. Seven-day-old soybean seedlings were exposed to 0.5 mM $CaCl_2$ solutions containing 25 μM Cd^{2+}, 10 μM La^{3+}, 1 μM Cu^{2+} and 30 μM Al^{3+} (pH 4.5). The 0–1 cm root apices were excised after 4 h Al treatment to study gene expression. Data are represented as means±SD of three biological replicates. Different letters above column represented significantly different ($p < 0.05$, *t*- test)

Consistent with the Jiyu70 roots, the soybean hairy roots showed lower expression of *GmSTOP1a* at pH 4.5 and increased expression at pH 3.5 or pH 5.5 (Fig. 2a; Fig. 10a). *GmSTOP1a* showed increased expression in *GmSTOP1a*–OE hairy roots under the three pH conditions (Fig. 10a), resulting in higher expression of *GmSTOP1c* (Close homolog to *AtSTOP2*) (Fig. 10b) and *GmCIPK23* (Close homolog to AtCIPK23) (Fig. 10c) at pH 3.5 but had a negligible effect on the expression of either *GmPGIP1* (Close homolog to *AtPGIP1*) (Fig. 10d) or *GmGDH1* (Close homolog to *AtGDH1*) (Fig. 10e). The expression of *GmPGIP1* was even inhibited at pH 5.5 in *GmSTOP1a*–OE hairy roots Complex regulation might occur for *GmSTOP1a*, *GmSTOP1c* or other genes putatively involved in pH stat regulation under different pH conditions.

Heterologous overexpression of *GmSTOP1a* in Arabidopsis

The root growth of col-4 Arabidopsis ecotype was inhibited by 55% under Al stress. The *GmSTOP1a*-OE

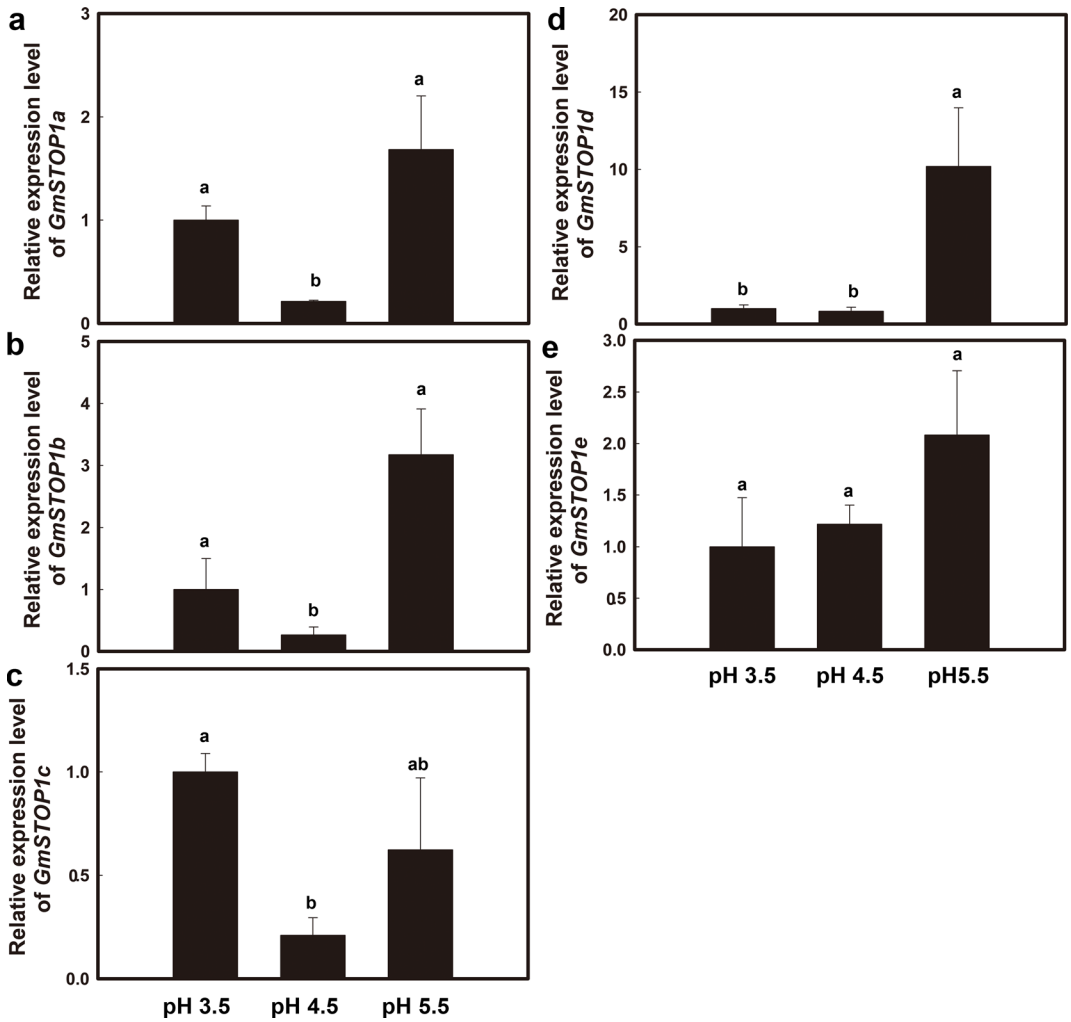

Fig. 4 Transcriptional expression of five *GmSTOP1* genes in response to different pH conditions. Seven-day-old soybean seedlings were exposed to 0.5 mM $CaCl_2$ solution (pH 3.5, 4.5 and 5.5). The 0–1 cm root apices were excised after 4 h in three kinds of pH treatment to study temporal expression. The 0–1 cm root segments were also excised from 4 h Al-treated or control soybean roots to study gene expression. The expression levels of *GmSTOP1a* (**a**), *GmSTOP1b* (**b**), *GmSTOP1c* (**c**), *GmSTOP1d* (**d**) and *GmSTOP1e* (**e**) were examined by qRT-PCR, with β-tubulin as the reference gene. Data are represented as means ±SD of three biological replicates. Different letters above column represented significantly different ($p < 0.05$, *t*-test)

Arabidopsis lines displayed slight alleviation of root growth compared with the col-4 ecotype, with 45% and 52% inhibition rate response respectively (Fig. 11a, c). *GmSTOP1a*-OE line 1 exhibited significantly higher RRE than WT under Al stress (Fig. 11c). The mutant of *atstop1* showed more sensitivity to Al stress with an RRE of 20% (Fig. 11b, c). The complementary expression of *GmSTOP1a* in *atstop1* produced two homogenous *GmSTOP1a*-CE lines with RRE of approximately 70% under Al stress, indicating the partial restoration of root growth (Fig. 11b, c).

Two Arabidopsis *GmSTOP1a*-OE lines displayed similar root growth as Arabidopsis col-4 under a pH condition of 4.2, 4.5 or 5.5 (Fig. 12a, b). The *atstop1* mutant showed sensitivity to pH 4.2, with significant inhibition of root growth compared with Arabidopsis ecotype (Fig. 12a, c). Two lines of complementary expression *GmSTOP1a* in the *atstop1* mutant (*GmSTOP1a*-CE) could significantly alleviate root growth under the three pH conditions consistent with the levels of wild-type Arabidopsis (Fig. 12a, c). Thus, *GmSTOP1a* can effectively restore the function of *AtSTOP1* in regulating pH tolerance.

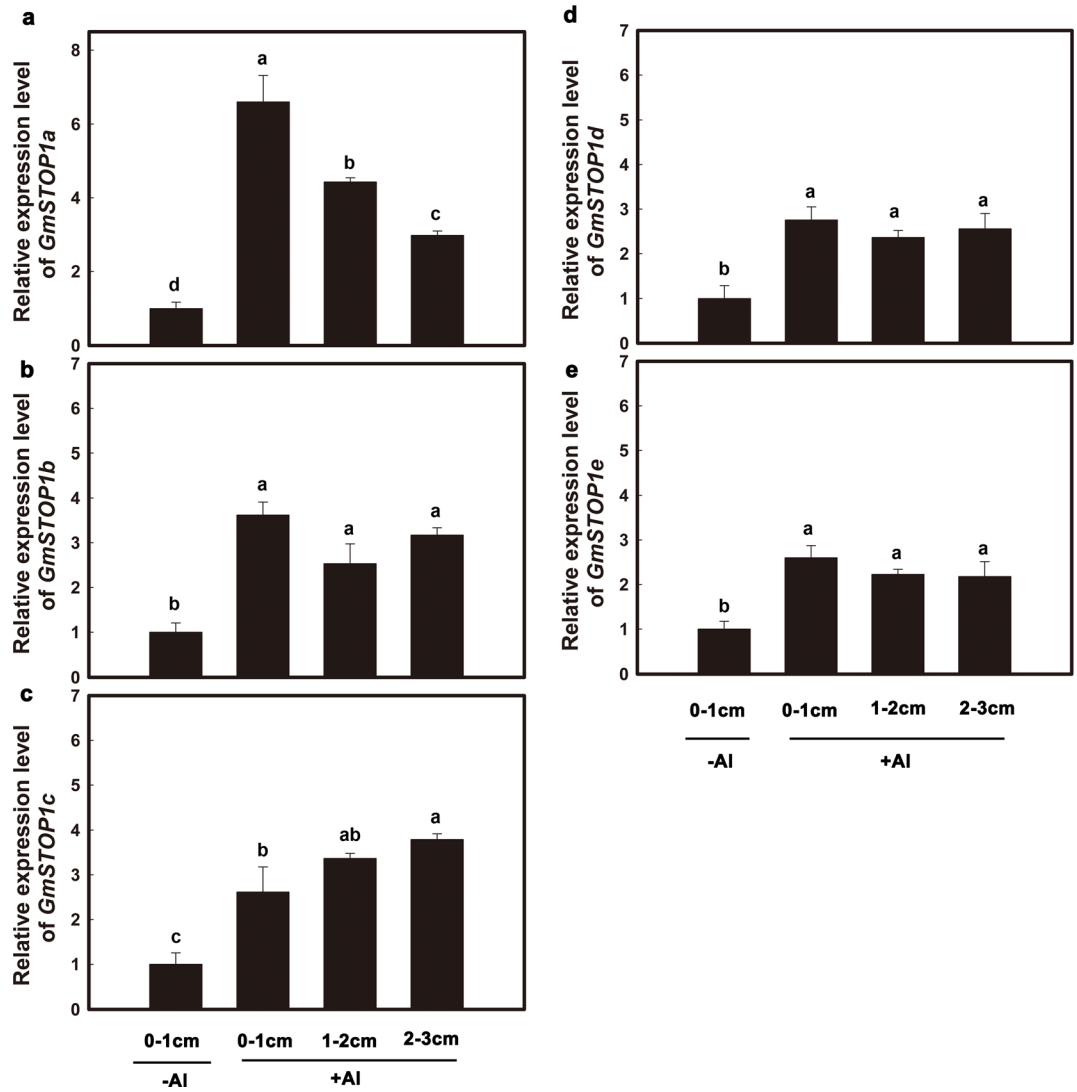

Fig. 5 Transcriptional expression of five *GmSTOP1* genes in soybean different root segments under Al stress. Seven-day-old soybean seedlings were exposed to 0.5 mM $CaCl_2$ solution containing 30 μM $AlCl_3$ (pH 4.5). The 0–1 cm root apices were excised at 0 h, and 0–1, 1–2, and 2–3 cm root segments were excised at 4 h after Al exposure.. The expression levels of *GmSTOP1a* (**a**), *GmSTOP1b* (**b**), *GmSTOP1c* (**c**), *GmSTOP1d* (**d**) and *GmSTOP1e* (**e**) were examined by qRT-PCR, with *β-tubulin* as the reference gene. Data are represented as means±SD of three biological replicates. Different letters above column represented significantly different ($p < 0.05$, *t*- test)

Discussion

C2H2 type zinc finger proteins were associated with different signal transduction pathways and participated in several cellular processes, thus regulating responses to multiple abiotic stresses (Kiebowicz-Matuk 2012). C2H2 type STOP1 transcription factor has been demonstrated to regulate pH and Al stress responses by controlling multiple genes in Arabidopsis (Iuchi et al. 2007; Sawaki et al. 2009). In the present study, five STOP1 homologs in soybean harbored the typical four zinc finger domains (Fig. 1a), were nuclear localized (Fig. 7a, b, c, d and e) and exhibited transactivation activities (Fig. 8). The five *GmSTOP1* genes were constitutively expressed in soybean plants but differed in transcriptional expression in response to Al stress, other metals and pH conditions (Figs. 2, 3and 4). With highest similarity to *AtSTOP1* or *VuSTOP1* in sequence, *GmSTOP1a* was

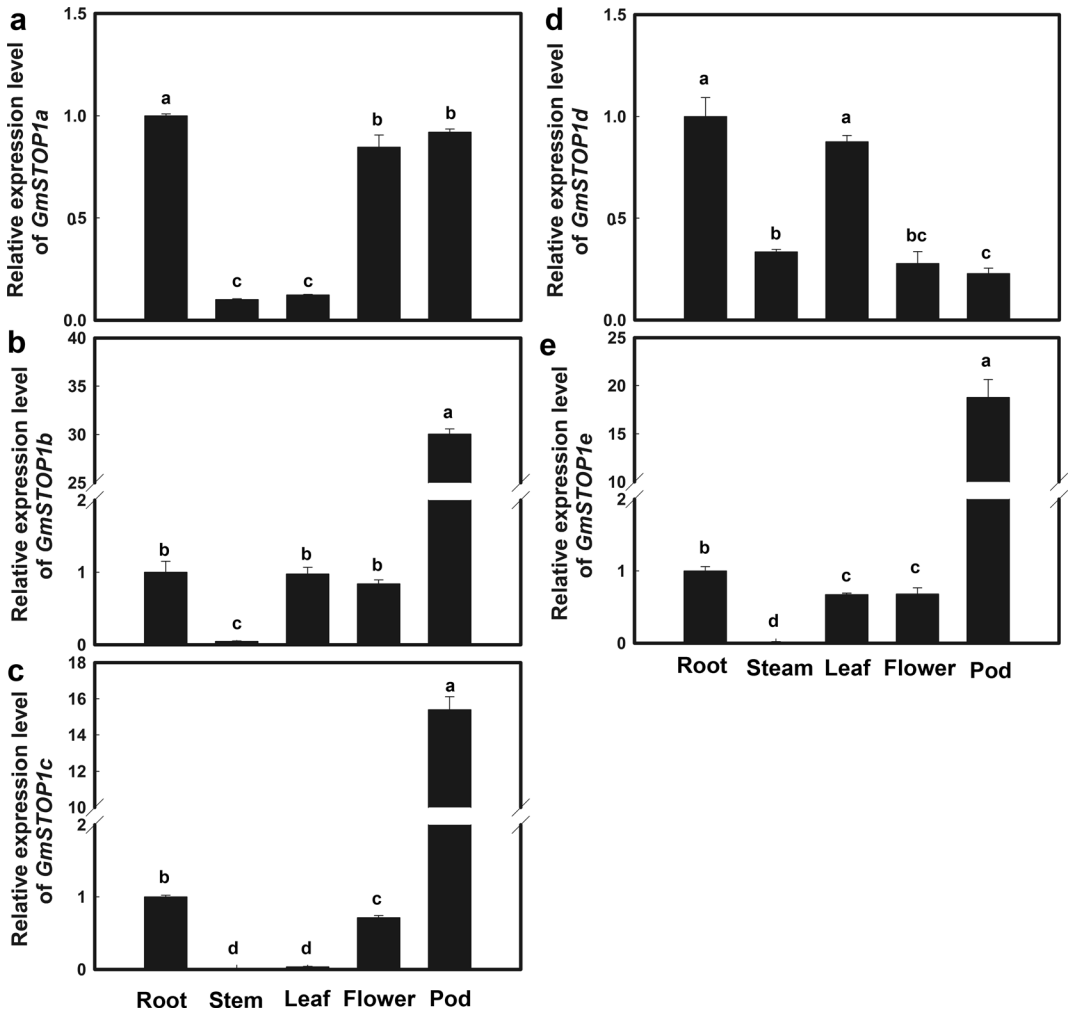

Fig. 6 Spatial expression of five GmSTOP1-like genes in soybean. After eighty days of growth in the field, the roots, shoots, leaves, flowers and pods were sampled from Jiyu70 soybean seedlings. The expression levels of *GmSTOP1a* (**a**), *GmSTOP1b* (**b**), *GmSTOP1c* (**c**), *GmSTOP1d* (**d**) *and GmSTOP1e* (**e**) were examined by qRT-PCR, with *β-tubulin* as the reference gene. Data are represented as means ±SD of three biological replicates. Different letters above column represented significantly different ($p < 0.05$, *t*- test)

selected to further characterized by by overexpression in soybean hairy roots (Figs. 9and 10), heterologous overexpression in wild-type Arabidopsis and in planta complementation expression in the *atstop1* mutant (Figs. 11 and 12).

Phylogenetic analysis showed that STOP1 proteins in dicots were clearly distinguished from those of monocots (Fig. 1b). AtSTOP2, a minor isoform of AtSTOP1 (Kobayashi et al. 2014), was completely distinct from AtSTOP1 in sequence (Fig. 1b). Five GmSTOP1 proteins shared four highly conserved C2H2 zinc-finger domains (Fig. 1a). GmSTOP1a displayed amino acid sequence similarity with VuSTOP1, AtSTOP1 and NtSTOP1, GmSTOP1b, GmSTOP1c, GmSTOP1d, and GmSTOP1e clustered more closely with AtSTOP2 (Fig. 1b). All five GmSTOP1 proteins localized to the nucleus (Fig. 7a, b, c, d and e) and showed transactivation activities (Fig. 8a, b, c, d and e). All GmSTOP1 proteins are the candidate transcription factors for regulating other genes to adapt to Al toxicity and/or pH stress.

GmSTOP1s had distinct regulation characteristics compared with *AtSTOP1 (*various Al or pH treatment cause no significant changes of STOP1 expression; Iuchi et al. 2007*)* and *OsART1* (the expression level was not affected by Al; Yamaji et al. 2009). But, the transcriptional expression of *GmSTOP1s* showed similar temporal

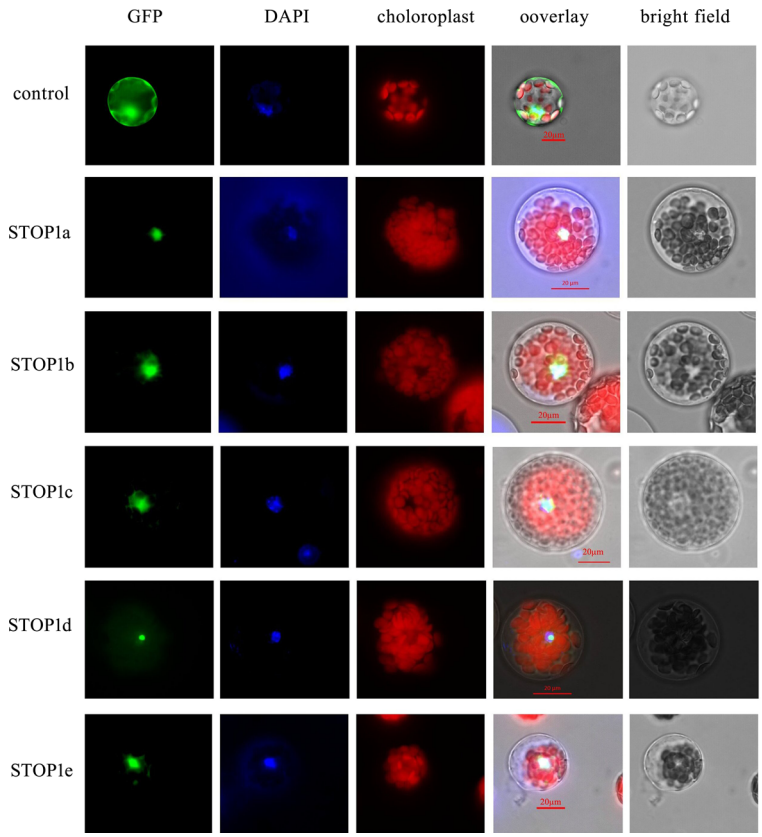

Fig. 7 Subcellular localization of STOP1 and homologous proteins. Subcellular localization of STOP1 and homologous proteins was determined by the transient expression of the GFP::STOP1 fusion proteins in Arabidopsis protoplasts cells. The images on the top row show the GFP-only control. The images on the lower row show the fusion proteins prepared with cDNA from soybean Jiyu70 (GFP::STOP1). The panels show fluorescence to detect GFP, chloroplast images, bright field, nuclear dye and an overlay of the four fields. Scale bar = 20 μm

pattern with *TaSTOP1*-A (wheat, Garcia-Oliveira et al. 2013) and increase aptitude as *VuSTOP1* (rice bean, Fan et al. 2015) under Al stress. All five *GmSTOP1* genes constitutively expressed in soybean roots, but were further increased in the transcription abundance by Al treatment (Fig. 2a, b, c, d, e). The temporal expression of the five *GmSTOP1* genes showed similar patterns of induction from 2 h, peaking at 4 h and returning to the basal level in the remaining Al treatment duration (Fig. 2a, b, c, d and e). Similarly, *TaSTOP1*-A transcript expression was found in the root tissues of Al-resistant wheat genotype (Barbela 7/72/92), with a slight induction (within the two hours of Al exposure), followed by a return to basal levels (Garcia-Oliveira et al. 2013). The aptitude of Al increased transcriptional expression of *GmSTOP1a* (Fig. 2a) is similar to *VuSTOP1*, whose expression is around 3–10 folds by 4 h Al stress in a dose-dependent manner (Fan et al. 2015). Recently, we also found the great increase of transcription abundance of *SbSTOP1* in sweet sorghum under Al stress (around 10 folds at 4 h) (Huang et al. 2018). Similar to *VuSTOP* in rice bean (Fan et al. 2015; higher expression at pH 4.0 compared with lower or higher pH conditions), the transcriptional expression of *GmSTOP1s* also fluctuated within different pH conditions (Fig. 4), and *GmSTOP1a*, *GmSTOPb*, *GmSTOP1c* showed higher expression at pH 3.5 or 5.5 (Fig. 4a, b and c). The transcription

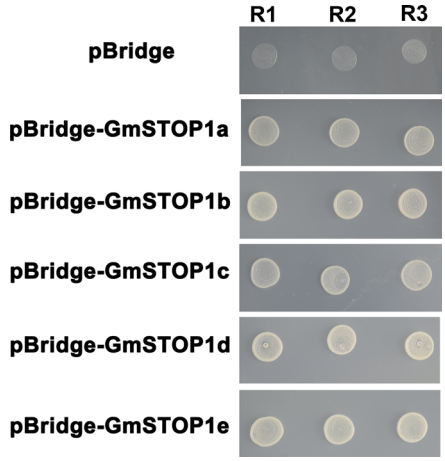

Fig. 8 Transactivation activity of GmSTOP1-like protein. The pBridge vector as a negative control could not grow on SD-Trp-His medium. The protein of pBridge-GmSTOP1a, pBridge-GmSTOP1b, pBridge-GmSTOP1c, pBridge-GmSTOP1d and pBridge-GmSTOP1e could grow on SD-Trp-His medium. R1–3 represented replicate 1,replicate 2 replicate 3 respectively

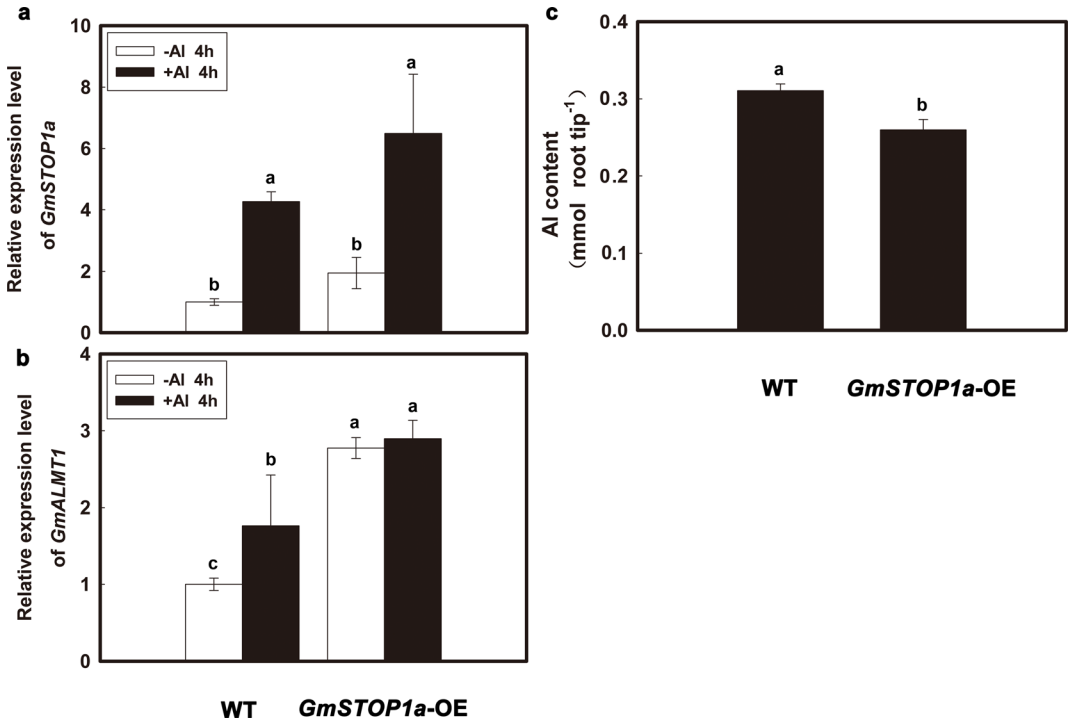

Fig. 9 The transcriptional expression in GmSTOP1a-OE hair roots under Al stress. a The transcriptional expression of *GmSTOP1a* was examined by qRT-PCR, with *β-tubulin* as the reference gene **b** The transcriptional expression of *GmALMT1* was examined by qRT-PCR, with *β-tubulin* as the reference gene. The gene transformation and treatment procedure was described in the Material and Methods. Data are represented as means ±SD of three biological replicates. Different letters above column represented significantly different ($p < 0.05$, *t*- test). **c** Ten 0–1 cm root apices were excised at 4 h for Al concentration measurements

abundance of *GmSTOP1s* fluctuated within root segments under Al stress (Fig. 5). Root apex is the most sensitive part of the root to Al^{3+} (Kochian et al. 2004). *GmSTOP1a* and *GmSTOP1e* showedobviously higher expression in the root apices (0–1 cm) than the basal root segments (1–2 cm, 2–3 cm) (Fig. 5a, e), closely related to Al toxicity from tissue localization. The five *GmSTOP1* genes were also constitutively expressed in the stem, leaf, flower and pod (Fig. 6). The highest expression of *GmSTOP1e* was found in the pod (Fig. 6a, b, c, d and e). The transcriptional expression of *GmSTOP1s* also responded to some metals, such as Cu^{2+} stress (Fig. 3). Recently, new functions other than proton and Al resistance have been reported in STOP1-like protein. For example, STOP1 protein was also implicated in the root developmental response to phosphorus deficiency in Arabidopsis (Mora-Macías et al. 2017). The same strategy is frequently recruited by plants for resistance to metal stress. In soybean, Al-induced citrate efflux is an important Al-resistance mechanism (Yang et al. 2000; Yang et al. 2001). Some metals, such as Cu^{2+}, can also induce minor citrate efflux (Nian et al. 2004). *GmSTOP1* might also be involved in other unknown functions, such as Cu^{2+} stress.

Either repression (*EguSTOP1*, Sawaki et al. 2014) or complementary expression (*CsSTOP1*, *LjSTOP1*, *PnSTOP1*, Ohyama et al. 2013; *VuSTOP1*, Fan et al. 2015; *SbSTOP1*, Huang et al. 2018) or both (*AtSTOP1*, Iuchi et al. 2007, Iuchi et al. 2009; Sawaki et al. 2009; *OsART1*, Yamaji et al. 2009; *PpSTOP1,* Ohyama et al. 2013) techniques were applied to evaluate the functions of *STOP1/ART1* type genes. Actually, overexpression technique was also helpfu for full evaluation of gene functions. For example, Larsen et al. (2007) reported that the mutant of *atals1* or *atals3* was hypersensitive to Al stress, but expression of each or both in yeast didn't affect yeast growth or Al uptake. Overexpression of each in Arabidopsis didn't confer higher root growth in comparison to WT with free or chelated $AlCl_3$. Thus, present study applied overexpression in soybean hairy roots, Arabidopsis ecotype and complementary expression in *atstop1* mutant to full evaluate the function of *GmSTOP1a* under Al or low pH conditions.

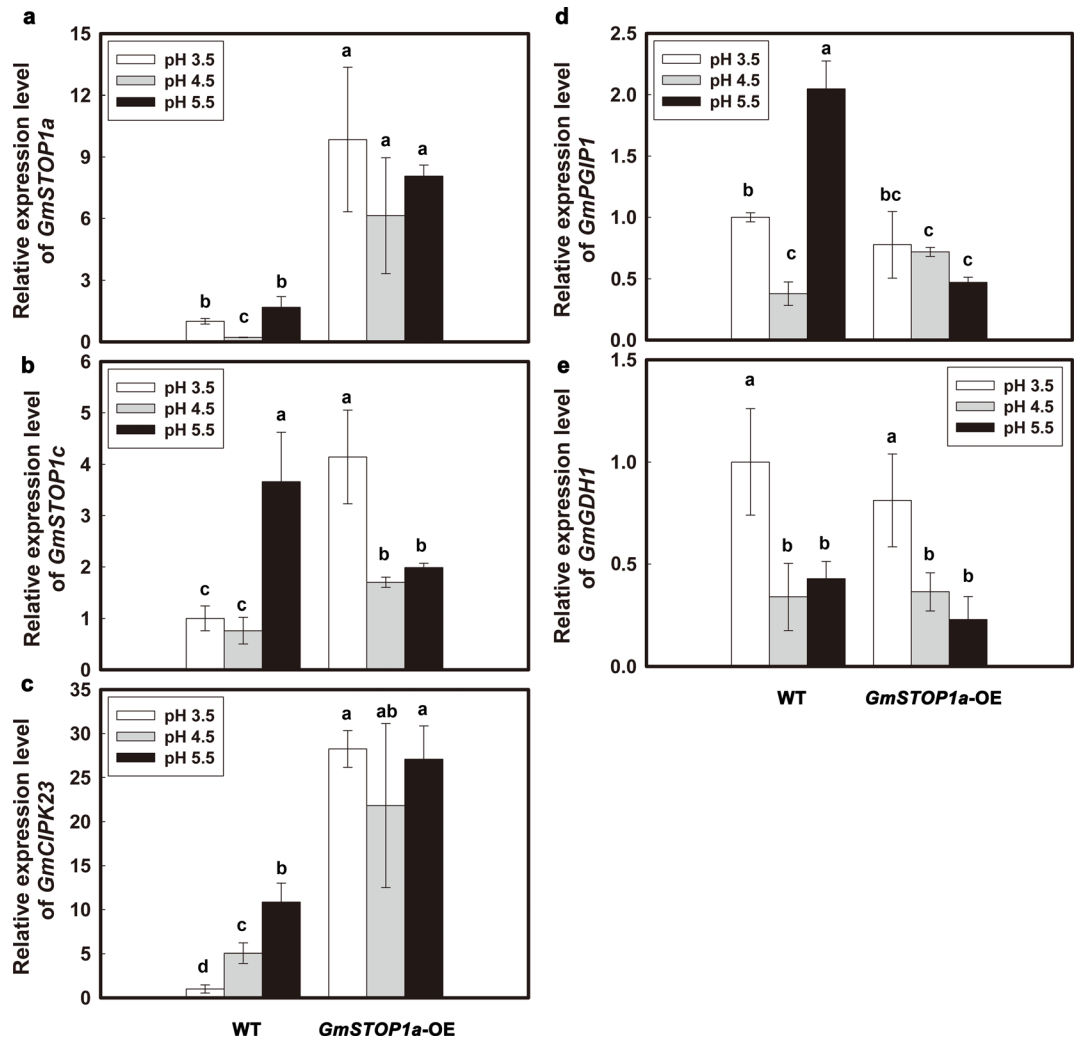

Fig. 10 The transcriptional expression in GmSTOP1a-OE hairy roots in response to different pH conditions. The expression levels of *GmSTOP1a* (**a**), *GmSTOP1c* (**b**), *GmCIPK23* (**c**), *GmPGIP1* (**d**) and *GmGDH1* (**e**) were examined by qRT-PCR, with β-tubulin as the reference gene. The gene transformation and treatment procedure was described in the Material and Methods. Data are represented as means ±SD of three biological replicates. Different letters above columns represented significantly different ($p < 0.05$, *t*- test)

GmALMT1, encoding the Al-activated malate transporter, is coordinately affected by low phosphorus, Al toxicity and low pH and is the first etablished gene associated with Al resistance in soybean (Liang et al. 2013). The regulation of the *ALMT1* gene by STOP1 has been reported as a conserved Al-resistance mechanism in Arabidopsis (Liu et al. 2009; Sawaki et al. 2014). The Al activation of *AtALMT1* expression is completely suppressed in the dysfunctional mutant of *atstop1* (Iuchi et al. 2007). Computation and in vitro binding assays showed that the promoter region of *AtALMT1* contains the STOP1 binding site and is thus involved in STOP1 regulation and Al induction (Tokizawa et al. 2015). In the present study, overexpression of *GmSTOP1a* in soybean hairy roots increased the expression of *GmALMT1* under –Al or + Al conditions, and decreased Al concentration indicating acquisition of Al resistance (Fig. 9a, b and c), indicating the regulation of *GmALMT1* by STOP1 transcription factor was also conserved in soybean.

The pH regulation mechanism is not completely understood. The Arabidopsis *atstop1* mutant showed great H^+ sensitivity (Iuchi et al. 2007), which was attributed to the down-regulation of a series of genes (Sawaki et al. 2009). For example, CBL-INTERACTING PROTEIN

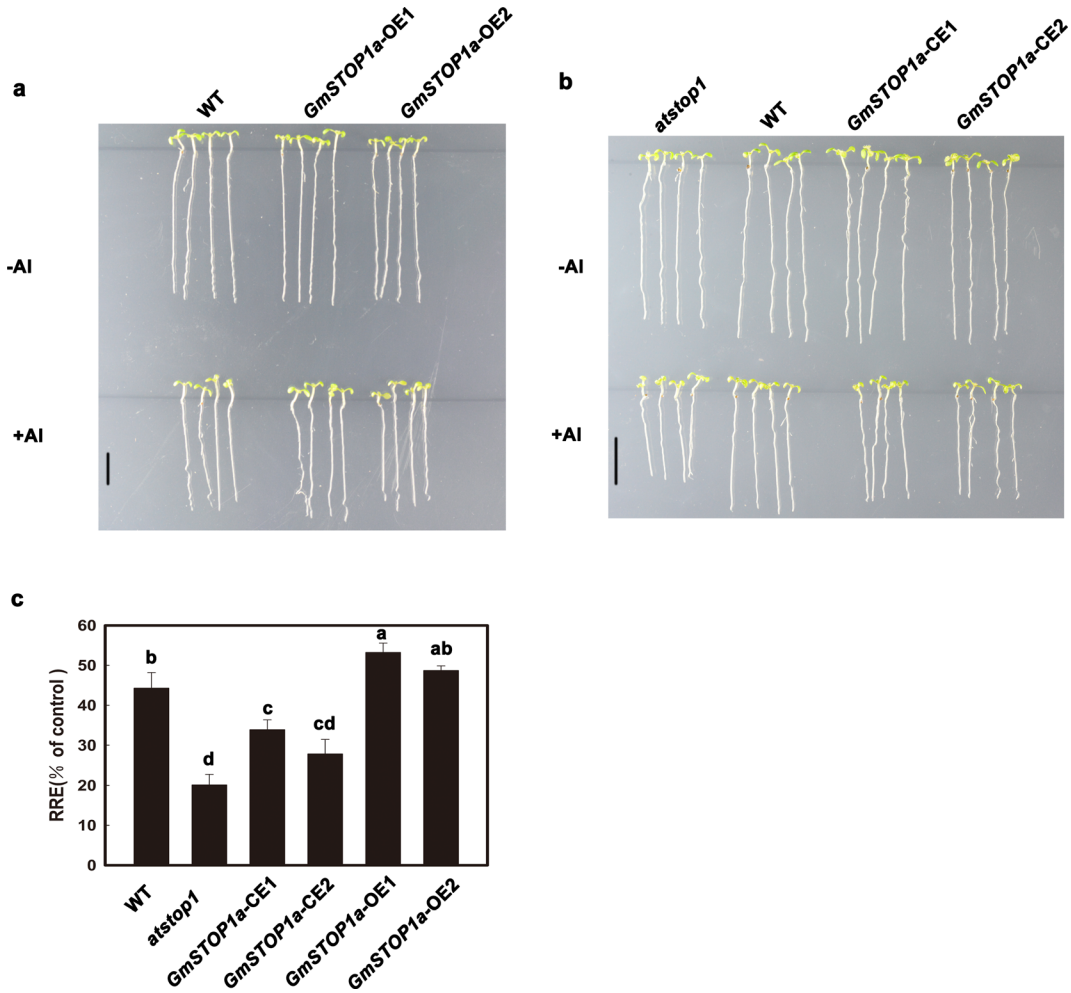

Fig. 11 Phenotype of Al resistance of Arabidopsis *atstop1* mutants complementarily expressing *GmSTOP1a* under Al stress. a Phenotypic analysis of WT, *GmSTOP1*-OE1 and *GmSTOP1*-OE2 under Al stress.Over-expression of *GmSTOP1a* in arabidopsis produced *GmSTOP1a*-OE lines. **b** Phenotypic analysis of *atstop1*, WT, *GmSTOP1a*-CE1 and *GmSTOP1*-CE2 under Al stress. Complementary expression *GmSTOP1a* in the *atstop1* mutant produced *GmSTOP1a*-CE lines. The bars represent the means ± SD, $n = 15–20$. Different letters above columns represented significantly different ($p < 0.05$, *t*- test). **c** Relative root elongation of *atstop1*, WT, *GmSTOP1a*-OE1, *GmSTOP1a*-OE2, *GmSTOP1a*-CE1 and *GmSTOP1a*-CE2 under Al stress. Scale bar = 5 mm. The gene transformation and treatment procedure was described in the Material and Methods. The bars represent the means ± SD, n = 15–20. Different letters above columns represented significantly different ($p < 0.05$, *t*- test)

KINASE23(*CIPK23*), GLUTAMATE DEHYDROGENASE1 (*GDH1*), and POLYGALACTURONASE-INHIBITING PROTEIN1 (*PGIP1*) were, respectively, implicated in ion transport, nitrogen metabolism, and pectin modification and subsequently cell wall function (Sawaki et al. 2009). These genes were down regulated in Arabidopsis *stop1* mutant and suggested to directly or indirectly regulate the low pH tolerance mechanism of STOP1 regulation (Sawaki et al. 2009). Their homologous genes were searched in soybean genomes and their transcriptional expression was studied in the soybean hairy root *GmSTOP1a*-OE lines (Fig. 10). CaMV 35S prompted *GmSTOP1a* overexpression increased the expression of *GmSTOP1c* (Fig. 10b) and *GmCIPK23* (Fig. 10c) at pH 3.5, but had negligible effect on *GmPGIP1* and *GmGDH1* at the three pH conditions of 3.5, 4.5 and 5.5 (Fig. 10d, e). Complex regulation might occur for *GmSTOP1a*, *GmSTOP1c* or other genes putatively involved in pH stat regulation under different pH conditions (Fig. 10).

Consistently, complementary expression of *GmSTOP1a* partially restore the root growth in *atstop1*

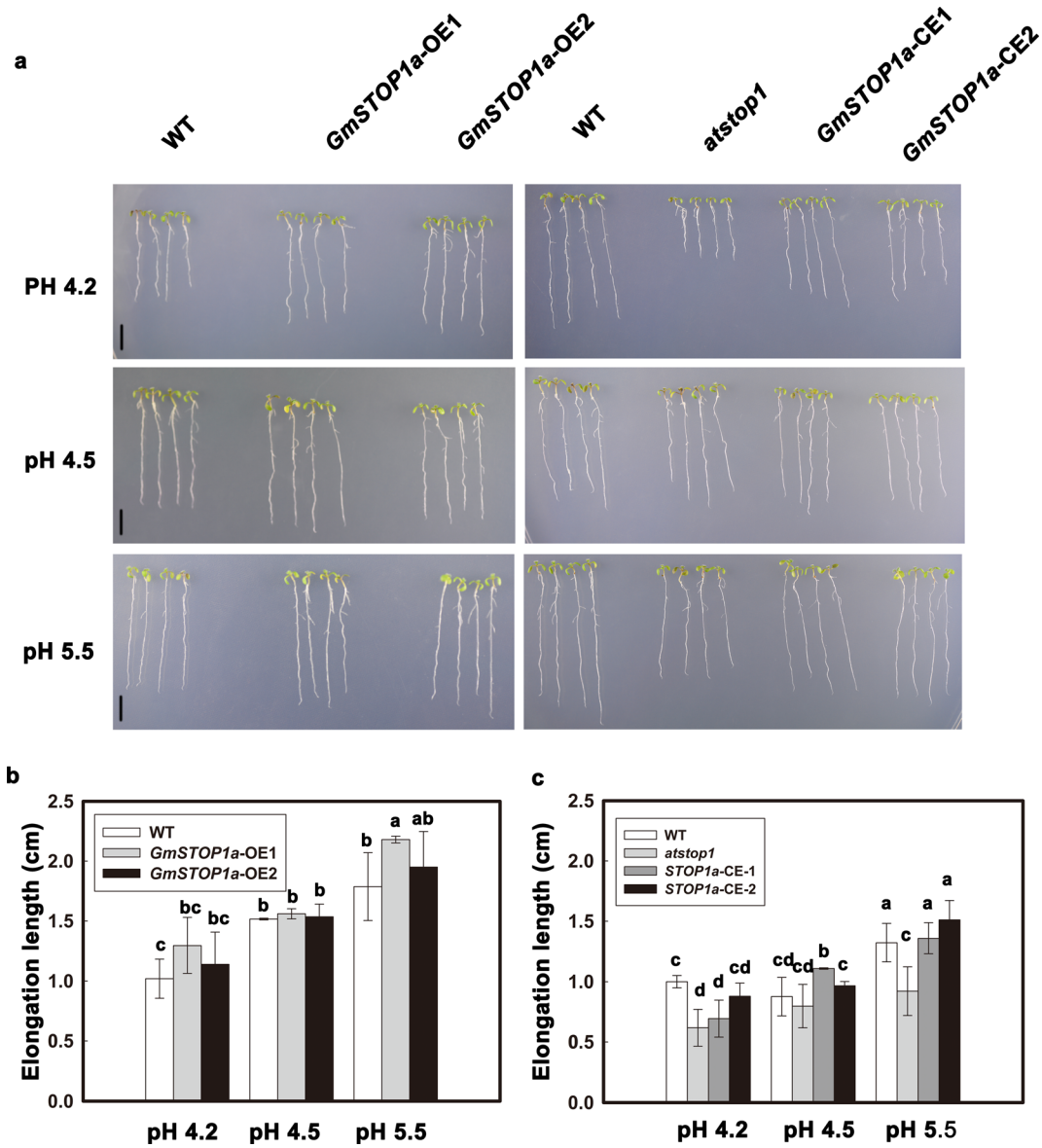

Fig. 12 Phenotype of Al resistance of Arabidopsis *atstop1* mutants complementarily expressing *GmSTOP1a* under different pH conditions. a Phenotypic analysis of *atstop1*, WT, *GmSTOP1a*-OE1, *GmSTOP1a*-OE2, *GmSTOP1a*-CE1 and *GmSTOP1a*-CE2 under pH stress. **b** Relative root elongation of WT, *GmSTOP1a*-OE1 and *GmSTOP1a*-OE2 under pH stress. The bars represent the means ± SD, n = 15–20. Different letters above columns represented significantly different ($p < 0.05$, *t*- test). **c** Relative root elongation of *atstop1*, WT, *GmSTOP1a*-CE1 and *GmSTOP1*-CE2 under pH stress. Scale bar = 5 mm. The gene transformation and treatment procedure are described in the Material and Methods. The bars represent the means ± SD, n = 15–20. Different letters above columns represented significantly different ($p < 0.05$, *t*- test)

under Al stress (Fig. 11b, c), and almost fully restored the pH sensitivity of *atstop1* (Fig. 12a, c). Thus, GmS*TOP1a* was suggested to exhibits the partial function of *AtSTOP1* in regulating both Al and low pH resistance in soybean.

Despite of the great increase of expression of Al resistance or pH regulation genes in *GmSTOP1a*-OE soybean hairy roots lines, the overexpression of *GmSTOP1a* in Arabidopsis produced slight alleviation or indiscernible effect onits Al (Fig. 11a, c) or low pH (Fig. 12a, b) resistance. Different from *GmSTOP1s* with Al increased expression in soybean (Fig. 2), the transcriptional abundance of *AtSTOP1* in Arabidopsis kept

constant under various Al or pH treatments (Iuchi et al. 2007). The addition of *GmSTOP1a* in arabidopsis can't produce further alleviating effect under Al or low pH stress (Figs. 11a, c and 12a, b), indicating that the existed of *AtSTOP1* in Arabidopsis ecotype might be sufficient to regulate the downstream Al or low pH resistance genes, or more complex regulation mechanism might involved in the functions of STOP1 type transcription factors.

In conclusion, *GmSTOP1a* plays a role similar to that of *AtSTOP1* to contribute to both Al resistance and H^+ tolerance. The first identification of *GmSTOP1a* as a transcription factor will be useful for clarifying the downstream Al or H^+ resistance genes in soybean. The finding will also help the molecular breeding of soybean in adaption to acidic soils. The other four homologs also respond to both low pH and Al stress and act as transcription factors, but their roles need further clarified.

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