



## Article

# Aluminum-Specific Upregulation of *GmALS3* in the Shoots of Soybeans: A Potential Biomarker for Managing Soybean Production in Acidic Soil Regions

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**Abstract:** Aluminum (Al) toxicity in acidic soils is a global agricultural problem that limits crop productivity through the inhibition of root growth. However, poor management associated with the application of soil acidity amendments such as lime ( $\text{CaCO}_3$ ) in certain crop types can pose a threat to low-input farming practices. Accordingly, it is important to develop appropriate techniques for the management of crop production in acidic soils. In this study, we identified *ALS3* (*ALUMINUM SENSITIVE 3*) in soybeans (*Glycine max*, cultivar Toyomasari), which is highly expressed in the shoot under Al stress. *GmALS3* (*Glyma.10G047100*) expression was found to be Al-specific under various stress conditions. We analyzed *GmALS3* expression in the shoots of soybean plants grown in two different types of acidic soils (artificial and natural acidic soil) with different levels of liming and found that *GmALS3* expression was suppressed with levels of liming that have been shown to eliminate soil  $\text{Al}^{3+}$  toxicity. Using soybeans as a model, we identified a potential biomarker that could indicate Al toxicity and appropriate liming levels for soybeans cultivated in acidic soils.

**Keywords:** aluminum; *ALS3*; gene expression; *Glycine max*; liming; management; soil acidity

## 1. Introduction

Inadequate fertilizer management is a risk for agricultural ecosystems. Overuse of fertilizers can result in soil-related problems such as salinity, excess and imbalance of nutrients, etc [1]. On the other hand, adequate management of fertilizers can alleviate the stresses found in several soil types. For example, acidic soils ( $\text{pH} < 5.5$ ) contain various stress factors, including aluminum (Al) and proton ( $\text{H}^+$ ) rhizotoxicities and phosphorus (P) and calcium (Ca) deficiencies [2–7]. Most of these stress factors could be alleviated by the application of fertilizers, which can be expensive. However, this method would be challenging for several farmers in developing countries/regions dominated by acidic soils. For example, crop production in the northeast (NE) region of India is always lower than the national average because of soil acidity. About 80% of arable land in the NE region is acidic owing to its high rainfall; however, most local farmers are not able to apply sufficient fertilizers [8]. A solution to enable sustainable food production in such regions could be achieved by the development of new farming approaches that involve suitable soil management.

Both field and laboratory experiments have shown that the inhibitory effects of acidic soil can be ameliorated by the application of Ca fertilizers, including calcium carbonate (limestone) and gypsum.

Liming (application of  $\text{CaCO}_3$ ) reduces the amount of soluble Al in the soil solution and exchangeable Al in soil particles. In contrast, the application of gypsum reduces the attraction of rhizotoxic  $\text{Al}^{3+}$  to the plasma membrane surface [9,10]. This indicates that a reduction of  $\text{Al}^{3+}$  rhizotoxicity in acidic soil can be achieved by the application of Ca fertilizers. However, the application of Ca fertilizers is not feasible for low-input farming practices in developing countries because of its high cost [11,12]. Moreover, it has been reported that excess liming decreases the yield of crops (such as upland rice) grown in some soil types [13]. Excess liming induces soil mineral leaching, which can result in phosphorous (P) and manganese (Mn) deficiencies in the soil [13–15] and can inhibit Mg acquisition by plants [16,17]. Taken together, an optimal liming program would be required to establish sustainable production of crops in acidic soil regions of developing countries [18].

A combination of soil and plant analyses can be used to design appropriate fertilizer management protocols; however, accurate and convenient analytical procedures are required. Accurate procedures have been developed to evaluate major nutrients in soils and plants (e.g., available P levels in the soil (e.g., [19])). Handy procedures using electrical impedance are available to quantify nitrogen (N) and potassium (K) levels in crop plants [20,21]. Such procedures have not yet been developed for estimating Al stress in acidic soils. One possible solution would be the application of biomarkers to evaluate various stress factors. Yang et al. [22] developed a transcript biomarker that can estimate N status in maize, while Sadler et al. [23] identified a number of putative biomarkers associated with different sources of abiotic stress in tomato (drought, heat and salinity). These findings suggest that gene expression biomarkers could be useful for managing Ca levels in acidic fields.

Previous studies have found that several Al-tolerance genes are regulated by the zinc finger transcription factor SENSITIVE TO PROTON RHIZOTOXICITY1 (STOP1) [24]. Functional genomics analyses have shown that Al tolerance regulated by STOP1 is conserved in land plants, including bryophytes [25–31]. On the other hand, studies in the model plant *Arabidopsis* have demonstrated that several STOP1-regulated genes show a nearly specific response to Al. For example, *ALS3*, a major Al-tolerance gene whose transcription is regulated by STOP1, shows specific expression under Al-stressed conditions in *Arabidopsis*. Although the molecular mechanism of *ALS3* underlying Al tolerance has not yet been clarified, several interesting characteristics were identified [32]. *ALS3* transports UDP-glucose as a bacterial-type ABC transporter [26], which is rare in plants and is encoded as single copy gene in both rice (*STAR2*) and in the *Arabidopsis* genome. Most of the STOP1-dependent Al-tolerance genes [e.g., ALUMINUM ACTIVATED MALATE TRANSPORTER1 (*AtALMT1*) and MULTIDRUG AND TOXIC COMPOUNDS EXTRUSION (*AtMATE*)] [26] are only inducible in the roots, as these protein-encoding genes play important roles in Al excursion from the roots. On the basis of comparative microarray analyses, we previously demonstrated that *ALS3* is also inducible in the shoots of *Arabidopsis* and tobacco [33]; however, its role in Al tolerance in the shoot remains unexplored. Given that the expression of *ALS3* in the shoot is essentially specific to Al in these model plants, we hypothesized that identification of this system would be useful in monitoring the levels of Al toxicity in soybeans grown in acidic soils.

The soybean is a commercially important crop that is widely cultivated in acidic soils, including the NE region of India [12]. In the present study, the soybean was used as a model to analyze *GmALS3* expression in shoots grown in acidic soil with different levels of liming. Our analysis suggests that *GmALS3* expression in the shoot under Al stress could be a biomarker to determine Al toxicity in soybeans as well as to manage liming in acidic soil.

## 2. Materials and Methods

### 2.1. DNA/Protein Sequence, Phylogenetic Analyses

The nucleotide and protein sequences of *AtALS3* and other orthologs of *Arabidopsis* shoot Al-inducible genes (*AT5G38200*, *AT1G09350* and *AT5G06860*; [33]) were retrieved from Phytozome-12 (<https://phytozome.jgi.doe.gov/pz/portal.html>) and NCBI (<https://www.ncbi.nlm.nih>.

gov/gene) database. A search for ALS3 orthologs of different plant species (*Nicotiana tabacum*, *Oryza sativa*, *Triticum aestivum*, *G. max*, *Cicer arietinum*, *Zea mays*, *Medicago truncatula*, *Cajanus cajan*, *Phaseolus vulgaris*, *Vigna angularis*, *Vigna mungo*, *Vigna radiata* and *Vigna unguiculata*) was carried out using the Basic Local Alignment Search Tool (BLASTP) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>). Sequence identity was analyzed by using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). A phylogenetic tree was generated using Phylogeny.fr (<http://www.phylogeny.fr/index.cgi>) [34].

## 2.2. Hydroponic Culture

Soybean (*G. max* L. Merrill) cultivar Toyomasari seeds were surface-sterilized with 1% sodium hypochlorite for 2 min, rinsed with deionized water and kept for 2 days on moist filter paper in the dark at 25 °C in Petri dishes. Germinated seeds were supported on mesh dipped into magenta boxes (growing area of 39.51sq. cm) filled with 250 mL modified MGRL medium which enhances Al rhizotoxicity at the plasma membrane surface according to Gouy–Chapman–Stern model (2% nutrients in the presence of 200 µM CaCl<sub>2</sub> [35,36]). Seedlings were grown for 10 days at 25 °C under 12 h illuminations (photosynthetic photon flux density; 37 µmol/m<sup>2</sup>/s) per day. The initial pH was adjusted to 5.6, and the medium was renewed every 2 days. For the Al-concentration-dependence experiment, plants pregrown for 10days were transferred to a control solution (2% MGRL media with 0 µM AlCl<sub>3</sub> (pH 4.5 and 6.0)) and treatment solution (2% MGRL media containing 25, 50 or 100 µM AlCl<sub>3</sub> (pH 4.5)) for 24 h [28,33]. During the time-course experiment, plants pregrown for 10days were exposed to 2% MGRL media containing 50 µM AlCl<sub>3</sub> for 0, 6, 24, 48 or 72 h (pH 4.5). For the pH experiment, 10-day-old pregrown plants were exposed to 2% MGRL media with different pH (4, 4.5, 5, 5.5, 6, 6.5 or 7.0) for 24 h [26]. To evaluate the effect of nutrients on the expression of *GmALS3*, 10-day-old pregrown plants were exposed to 2% MGRL media deficient in specific nutrients (nitrogen (N), phosphorus (P) or potassium (K)) for 24 h. For the Al-specific experiment, plants pregrown for 10 days were exposed to 2% MGRL media containing rhizotoxic ions (50-µM AlCl<sub>3</sub>, 25-µM CdCl<sub>2</sub>, 10-µM CuSO<sub>4</sub> or 75-mM NaCl; pH 4.5; this condition inhibits relative root elongation by 30–50%) or no rhizotoxic ions (pH 4.5 or 6.0) for 24 h [33]. The relative root elongation of soybeans was measured as (net root growth with stress/net root growth without stress) cm × 100%. Plant tissues were harvested without touching the plants in order to avoid the likelihood of wounding. After harvesting, tissues were immediately frozen in liquid nitrogen and stored at −80 °C. These samples were subsequently utilized for RNA isolation and transcript analysis.

## 2.3. Acidic Soil Experiment

We used two types of acidic soils: an artificial acidic soil, with artificial neutral soil (PROTOLEAF, Tokyo, Japan) as a control and an acidic soil collected from NE region of India, with neutral field soil as a control. To evaluate soil acidity, the pH (1:2.5 w/v soil: water solution) and exchangeable Al content of the soil were quantified using the methods described by Koyama et al. [37]. Acidic soils were neutralized by adding CaCO<sub>3</sub> at levels that have been shown to eliminate Al<sup>3+</sup> toxicity [36] (5.0 g kg<sup>−1</sup> for artificial acidic soil and 6.2 g kg<sup>−1</sup> for acidic soil collected from the field). Next, 2 g kg<sup>−1</sup> CaCO<sub>3</sub> was added to the neutral soil as a mock treatment. Different legumes (*G. max* (cultivar Toyomasari, Kinshu, Tanbakuro and Tsurunoko), *C. arietinum*, *C. cajan*, *V. mungo*, *V. radiata* and *V. unguiculata*) were grown in individual plastic pots filled with 500 g of soil (dry weight). After sowing, the plants were grown for two weeks at the same temperature and light/dark regime as those used in hydroponics. Throughout the experiment, the plants were irrigated each day with deionized water to maintain soil moisture. For gene-expression analysis, plant tissues were harvested without directly touching the plants to avoid potential plant injury. After harvesting, the tissues were immediately immersed in liquid nitrogen and stored at −80 °C for subsequent RNA isolation and transcript analysis.

#### 2.4. RNA Extraction and cDNA Synthesis

Samples of shoot tissues (150 mg) were homogenized using a Shake Master Neo tissue homogenizer (Hirata, Shiga, Japan). Total RNA was isolated using Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) and a NucleoSpin RNA Plant Kit (Macherey–Nagel, Germany) according to the manufacturer's instructions. The integrity of the isolated total RNA was evaluated using agarose gels stained with ethidium bromide. The quality of the RNA was analyzed based on A260/A280 and 260/230 absorbance ratios by using a NanoVuePlus spectrophotometer (Biochrom, Holliston, MA, USA). Samples with an RNA integrity (28S/18S ribosomal RNA bands) less than 2.2, a 260/280 ratio of less than 1.9 or a 260/230 ratio of less than 1.8 were not used for the preparation of cDNA. Aliquots (500 ng) of total RNA were reverse-transcribed using ReverTra Ace quantitative PCR master mix (Toyobo, Osaka, Japan) and aPrimeScript™ 1ststrand cDNA Synthesis Kit (Takara, Japan) containing genomic DNA remover in a final reaction volume of 10 µL, following the manufacturers' instructions. Prior to usage, all cDNA samples were stored at −80 °C.

#### 2.5. Primer Design

The primers used to amplify all genes were designed using Primer 3 software (<http://bioinfo.ut.ee/primer3-0.4.0/>) (Table S1) according to Takara Bio real-time PCR primer design guidelines (<https://www.takara-bio.co.jp/prt/pdfs/prt3-1.pdf>). Briefly, the primer sets had a melting temperature of between 60 and 65 °C, GC content between 40% and 60%, a size between 17 and 25 bp, and amplicon sizes ranging from 80 to 150-bp in length [28]. The primers were synthesized by Eurofins Genomics K.K. (Tokyo, Japan).

#### 2.6. Transcript Analysis

A quantitative polymerase chain reaction (qRT-PCR) was performed using SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan) and SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) with a Dice Real Time System II MRQ thermal cycler (Takara, Japan) and CFX96 Real-Time system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Briefly, all quantifications were carried out based on the qRT-PCR standard curve method proposed by Bustin et al. [38], as described by Kobayashi et al. [39,40]. For all quantifications, a standard curve was constructed using a cDNA dilution series. The transcript levels of selected genes [*Glyma.10G047100* (*GmALS3*), *Glyma.03G175800*, *Glyma.13G044600*, *Glyma.05G124000*, *Glyma.03G222000*, *CaALS3*, *CcALS3*, *VmALS3*, *VrALS3*, *VuALS3* and *GmNRAMPs* (1a, 1b, 2b)] were normalized to those of the internal standards (Figure S1) (*GmUBQ10* [41], *CaActin* [42], *CcActin* [28], *VmActin* [43], *VrActin* [44] and *VuActin* [45]) and expressed as relative fold changes (RFCs) compared with the control [40,46]. We included a control with no reverse transcriptase to check for genomic DNA contamination. The amplification efficiencies of the primer pairs used in all qRT-PCR assays were found to be between 93.6% and 112.3% (Table S2) and the standard curve coefficients of determination ( $R^2$ ) were all greater than 0.98. A dissociation curve analysis (60 to 95 °C) was performed after 40 cycles to verify the specificity of the amplicons. The amplicon specificities were confirmed by the presence of a single peak. Each amplification was repeated three times. For semiquantitative RT-PCR, PCR products were separated by 3% agarose gel electrophoresis and visualized with GelRed staining. Gel images were obtained using a Typhoon 9410 image scanner (Amersham Biosciences, NJ, USA).

#### 2.7. Growth and Biomass Assay in Acidic Soil

Growth was measured in terms of shoot and root length, shoot and root fresh weight and shoot and root dry weight. The relative shoot and root elongation of soybeans grown in acidic soil for two weeks was measured as (net shoot or root growth in the acidic soil/net shoot or root growth in neutral soil) cm × 100%. After this two-week period, the plant shoots and roots were separated from the soil.

The fresh weight was measured immediately after incubation; thereafter, samples were oven-dried at 80 °C for 48 h in order to determine the dry weight.

### 2.8. Quantification of Al Content

Three sets of plants were grown in both acidic and non-acidic soil for two weeks, and the shoots and roots were excised and rinsed with deionized water. The samples were oven-dried at 80 °C for 48 h. The samples were then digested with concentrated HNO<sub>3</sub> (2 mL; electronic chemical grade; Kanto Chemical, Tokyo, Japan) and concentrated H<sub>2</sub>O<sub>2</sub> (0.5 mL; semiconductor grade; Santoku Chemical, Tokyo, Japan), and the digestate was dried using a Digi PREP digestion apparatus (SCP science, Baie-D'Urfe, QC, Canada). The samples were then solubilized in 3 mL of 2% (v/v) HNO<sub>3</sub> in ultrapure water. The shoot and root Al content were analyzed using inductively coupled plasma–mass spectrometry (ICP-MS; ELAN DRC-e; PerkinElmer, Waltham, MA, USA) according to the manufacturer's manual [47].

### 2.9. Statistical Analysis

The experimental design was completely randomized. All data are expressed as the mean (±standard error) of three biologic replicates (3–4 plants per replicate), and three technical replicates were run for each biologic replicate. Differences between the parameters were evaluated using Student's *t*-test with Microsoft Excel 2010, and *p*-values ≤ 0.05 were considered to be statistically significant.

## 3. Results

### 3.1. Identification of Orthologs of AtALS3 in Soybeans That Are Inducible by Aluminum

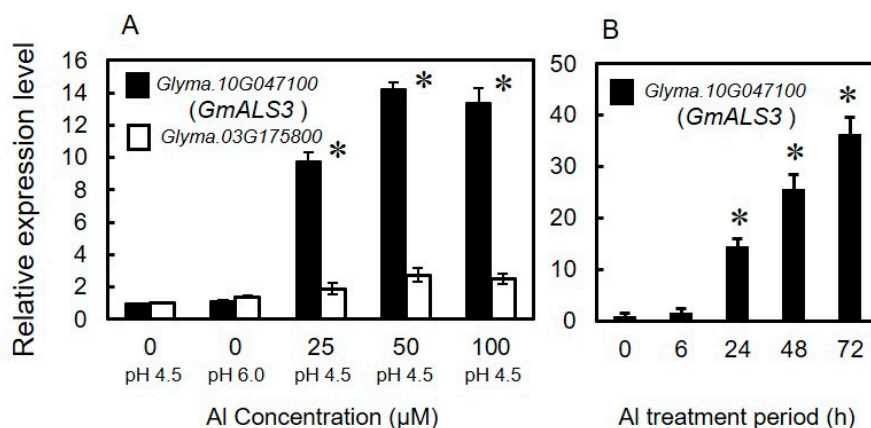
AtALS3 orthologs in soybeans were identified by the Phytozome 12 with BLASTP program. Based on the scores (S) and expected (E) values of BLASTP (i.e., the E-value decreases exponentially as the S of the orthologs increase), we identified two protein sequences (Table 1) that showed high similarity (90.1% and 89.7%) to AtALS3. We analyzed the expression levels of the genes encoding these orthologs in the shoots after exposing the roots to different concentrations of Al. Neither gene was induced by the low pH treatment (pH 4.5). By contrast, expression of *Glyma.10G047100*, but not *Glyma.03G175800* was significantly induced in the shoots (Figure 1A) during 24 h of Al treatment (Figure 1B). This suggests that *Glyma.10G047100* is the Al-responsive orthologue of AtALS3. Hereafter, *Glyma.10G047100* is designated as *GmALS3*.

**Table 1.** AtALS3 orthologs in soybeans.

Peptide Sequence ID ‡	Score §	E-Value ¶	Sequence		Description †
			Similarity €	Identity ±	
Glyma.10G047100	428.7	$6.2 \times 10^{-151}$	90.1	77.94	Putative ABC transport system permease protein
Glyma.03G175800	413.7	$7.7 \times 10^{-145}$	89.7	74.26	Putative ABC transport system permease protein

‡ peptide sequence ID, § BLAST score, ¶ expected value (E), € sequence similarity, † descriptions were retrieved from the Phytozome-12 database and ± sequence identity was analyzed by Clustal Omega.





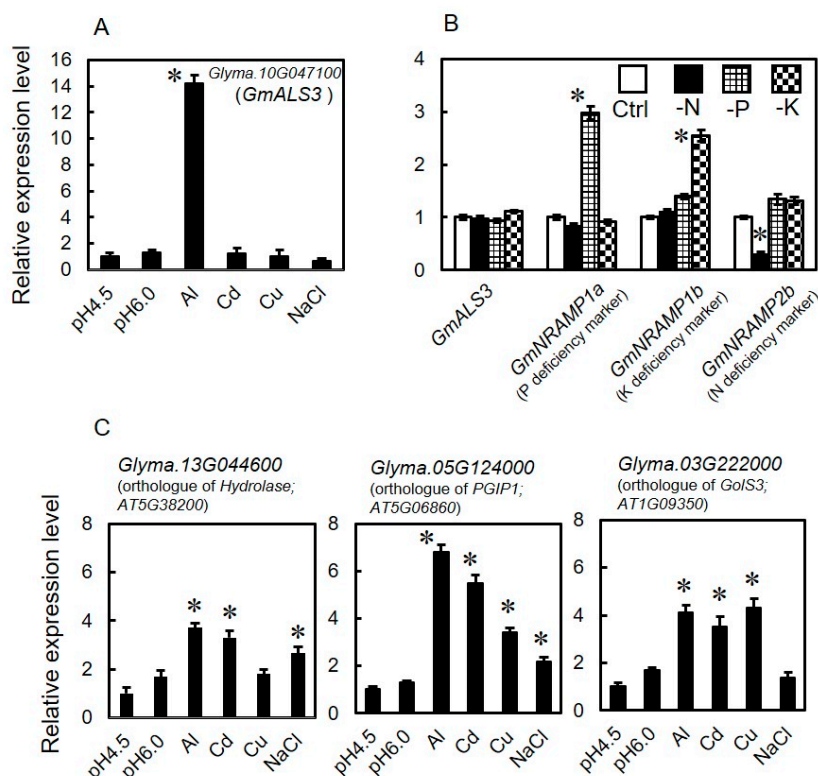
**Figure 1.** Expression of *AtALS3* orthologs in soybeans. Plants were pregrown for 10 and then transferred to  $-Al$  (0  $\mu M$   $AlCl_3$ , pH 4.5 and 6.0) or  $Al$ -rhizotoxic ( $AlCl_3$ , 25, 50 or 100- $\mu M$  (pH 4.5)) solutions. (A) Expression levels of *Glyma.10G047100 (GmALS3)* and *Glyma.03G175800* in the shoots were quantified after 24 h exposure of the roots to the  $Al$ -rhizotoxic or  $-Al$  (pH 4.5 and 6.0) solutions, asterisks indicate significant differences compare to  $-Al$  at pH 4.5 (Student's *t*-test,  $p < 0.05$ ); (B) expression level of *Glyma.10G047100 (GmALS3)* in the shoots of soybeans after exposure of the roots to  $Al$ -rhizotoxic solutions containing 50  $\mu M$  of  $AlCl_3$  (pH 4.5) at various time points, asterisks indicate significant differences compare to 0 h (Student's *t*-test,  $p < 0.05$ ). Expression level quantified using qRT-PCR using *GmUBQ10* as an internal standard. Values are mean  $\pm$ SE ( $n = 3$ ).

### 3.2. Expression Profile of *GmALS3* in Response to Rhizotoxic Ion Stress and Nutrient-Deficiency Treatments under Hydroponic Culture Conditions

The expression of *GmALS3* was profiled in the shoots after exposure of the roots to various rhizotoxic stress conditions (Al, Cd, Cu and NaCl). All of the ionic stress conditions induced similar levels of inhibition on the relative root elongation (about 30% of inhibition at 24 h, about 50% at 72 h; Figure S2A). After 24 h of treatment, the expression of *GmALS3* was specifically induced by Al in the shoots, but not by the others (Figure 2A). In addition, we also observed that pH alone had no significant effect on the expression of *GmALS3* (Figure S2B). These results suggest that expression of *GmALS3* is nearly specific to Al among rhizotoxic ion stresses.

To evaluate the responses of *GmALS3* to deficiency of macronutrients, we analyzed the expression of *GmALS3* in the shoots after exposure of the roots to solutions deficient in nitrogen (N), phosphorus (P) or potassium (K). In these experimental conditions, we found typical altered expression of marker genes of N, P and K deficiencies [48]. *GmNRAMP2b* (a repressive marker gene of N deficiency) was repressed in N deficiency. *GmNRAMP1a* (an inducible marker gene of P deficiency) and *GmNRAMP1b* (an inducible marker gene of K deficiency) were induced in P deficiency and K deficiency, respectively (Figure 2B). By contrast, the expression of *GmALS3* was comparable to that in the control solution containing all nutrients (Figure 2B). These results showed that *GmALS3* expression was induced by Al stress; other ion-related stresses or nutrient deficiency stress had no significant effect on the expression.

On the other hand, we also analyzed the Al-specific expression of other genes in soybean, which are orthologs of specifically Al-inducible genes in *Arabidopsis* [33] [*Glyma.13G044600* (orthologue of *Hydrolase*; AT5G38200), *Glyma.05G124000* (orthologue of *PGIP1*; AT5G06860) and *Glyma.03G222000* (orthologue of *GolS3*; AT1G09350)]. Although all of these genes were inducible by Al, they did not show an Al-specific response, as *GmALS3* did (Figure 2C). These observations suggest that *GmALS3* could be used to evaluate Al rhizotoxicity compared with other Al-inducible genes.

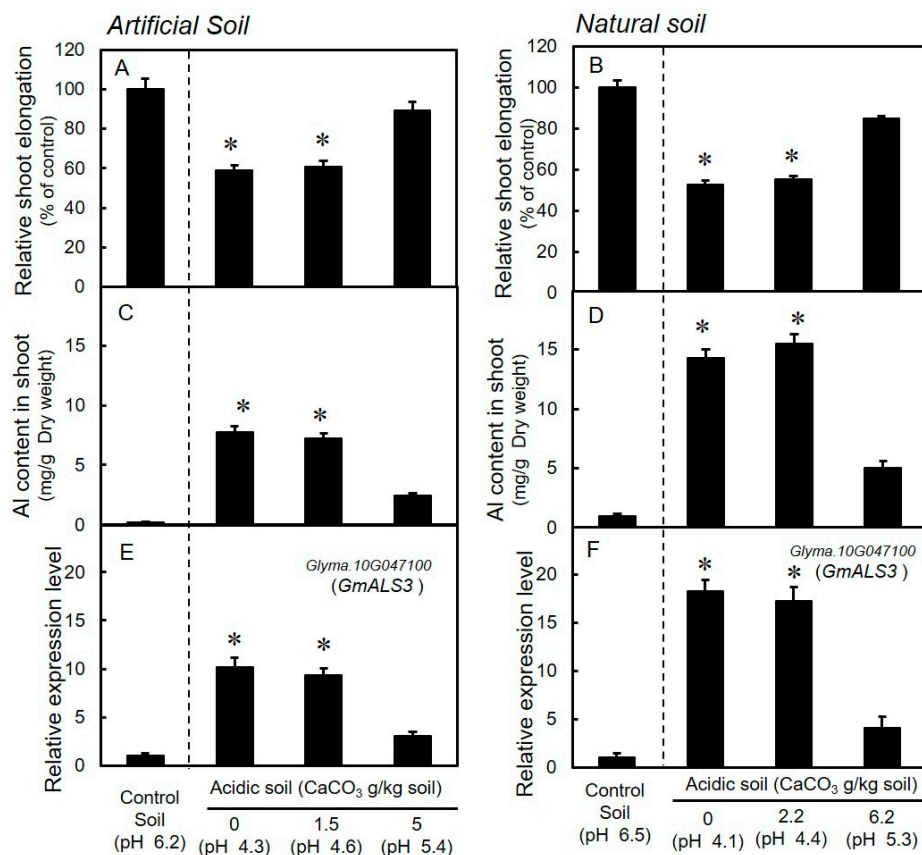


**Figure 2.** Specificity of the *GmALS3* (*Glyma.10G047100*) in response to Al rhizotoxicity in soybeans. (A) Expression of *GmALS3* in the shoots of 10-day-old pregrown soybean plants after exposure of the roots to without rhizotoxic ions (0  $\mu$ M Al, Cd, Cu or NaCl; pH 4.5 or 6.0) or with various rhizotoxic (50  $\mu$ M  $\text{AlCl}_3$ , 25  $\mu$ M  $\text{CdCl}_2$ , 10  $\mu$ M  $\text{CuSO}_4$  and 75 mM NaCl; pH 4.5; concentrations used in the root growth inhibition study; Figure S2A) solutions for 24 h; (B) *GmALS3* expression along with nutrient-responsive marker genes (*GmNRAMP1a*, *GmNRAMP1b* and *GmNRAMP2b*) in the shoots of 10-day-old pregrown soybean plants after exposure of the roots to nutrient solution with deficiencies in nitrate (-N), potassium (-K) or phosphate (-P) for 24 h (pH 6.0); (C) expression of other genes [orthologs of specifically Al-inducible genes in *Arabidopsis*; *Glyma.13G044600* (orthologue of *Hydrolase*; *AT5G38200*), *Glyma.05G124000* (orthologue of *PGIP1*; *AT5G06860*), *Glyma.03G222000* (orthologue of *GolS3*; *AT1G09350*)] in the shoots of 10-day-old pregrown soybean plants after exposure of the roots to without rhizotoxic ions (0  $\mu$ M Al, Cd, Cu and NaCl; pH 4.5 or 6.0) or with various rhizotoxic (50- $\mu$ M  $\text{AlCl}_3$ , 25- $\mu$ M  $\text{CdCl}_2$ , 10- $\mu$ M  $\text{CuSO}_4$  and 75 mM; pH 4.5) solutions for 24 h. Expression levels quantified using qRT-PCR. *UBQ10* was used as an internal standard. Data are presented as the means  $\pm$  SE ( $n = 3$ ). Asterisks indicate a significant difference (Student's *t*-test,  $p < 0.05$ ) from without rhizotoxic ions (pH 4.5) in panels (A) and (C) and control nutrient solution in panel (B).

### 3.3. Growth and Expression Levels of *GmALS3* in Soybeans Grown in Acidic Soils with Different Levels of $\text{CaCO}_3$

We analyzed the growth recovery and expression of *GmALS3* in the shoots of soybeans grown in acidic soils with different levels of  $\text{CaCO}_3$ . In this study, we used two types of acidic soils: an artificial acidic soil (pH 4.3, exchangeable Al  $31.2 \pm 0.3$ ) used for changing the blue color of hydrangea flowers (Al-dependent reaction) that is sold commercially in Japan and an acidic soil collected from the NE region of India (pH 4.1, exchangeable Al  $54.7 \pm 0.4$ ) (Table 2). Both acidic soils contained high amounts of exchangeable Al without  $\text{CaCO}_3$ , which was decreased by neutralizing the pH by addition of  $\text{CaCO}_3$  (Table 2). Toxic effects of Al in the soils were evaluated by two-week growth assay. A neutralization pH of about 4.5 did not show any recovery of growth in either acidic soil (Figure 3A,B, Figures S3 and S4; Tables S3 and S4). By contrast, the addition of  $\text{CaCO}_3$ , which neutralized the pH to above 5.3, almost recovered growth in both soils (Figure 3A,B, Figures S3 and S4; Tables S3 and S4). The growth

inhibition was associated with the accumulation of Al in the shoots (Figure 3C,D) and roots (Figure S5), which decreased as the application of  $\text{CaCO}_3$  increased. The accumulation of Al in the shoots derived from the high  $\text{CaCO}_3$  treatment (addition of 5 g and 6.2 g  $\text{CaCO}_3 \text{ kg}^{-1}$  soil) was significantly lower than that in shoots obtained from the low (1.5 and 2.2 g  $\text{CaCO}_3 \text{ kg}^{-1}$  soil) and no  $\text{CaCO}_3$  treatments (Figure 3C,D). Expression of *GmALS3* was induced in the acidic soils, but not in the neutral pH soils. *GmALS3* expression was correlated with the amount of exchangeable Al in the acidic soils. The expression level of *GmALS3* was comparable in the low and no  $\text{CaCO}_3$  treatments, but it was decreased when high levels of  $\text{CaCO}_3$  were applied to both acidic soils (Figure 3E,F). These results suggest that suppression of Al toxicity by  $\text{CaCO}_3$  for soybeans grown in acidic soils can be evaluated by *GmALS3* expression in the shoots.



**Figure 3.** Growth and expression levels of *GmALS3* in soybeans grown in artificial and natural soils with different levels of  $\text{CaCO}_3$ . Plants were grown for two weeks in neutral soil (addition of 2 g  $\text{CaCO}_3 \text{ g/kg}$  soil) as a control and in acidic soil with the addition of different levels of  $\text{CaCO}_3$  (0, 1.5 and 5 g/kg soil for artificial acidic soil; 0, 2.2 and 6.2 g/kg soil for natural acidic soil) as a treatment; (A) Relative shoot elongation of soybeans grown in artificial soils; (B) relative shoot elongation of soybeans grown in natural soils; (C) Al content of in the shoots of soybeans grown in artificial soils; (D) Al content in the shoots of soybeans grown in natural soils; (E) expression of *GmALS3* (*Glyma.10G047100*) in the shoots of soybeans grown in artificial soils; (F) expression of *GmALS3* (*Glyma.10G047100*) in the shoots of soybeans grown in natural soils. Al concentration in the shoot was quantified using ICP-MS. Expression levels quantified using qRT-PCR. *GmUBQ10* was used as an internal standard. Data given as mean and  $\pm$  SE ( $n = 3$ ). Asterisks indicate a significant difference (Student's *t*-test  $p < 0.05$ ) compared to control soil.

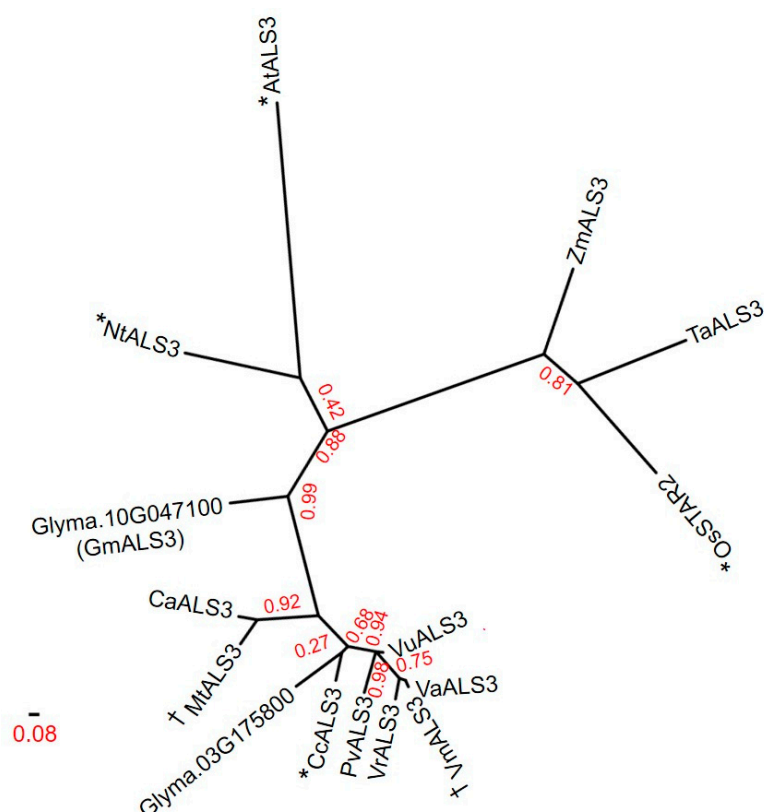


**Table 2.** Effect of CaCO<sub>3</sub> supply on pH and exchangeable Al in artificial and natural soils

Soil Type	Soil Condition	CaCO <sub>3</sub> (g kg <sup>-1</sup> soil)	Soil pH (H <sub>2</sub> O)	Exchangeable Al (meqkg <sup>-1</sup> soil)
Artificial	Neutral soil	2	6.2	3 ± 0.5
	Acidic soil	0	4.3	31.2 ± 0.3
	Acidic soil	1.5	4.6	27.5 ± 0.1
	Acidic soil	5	5.4	8.4 ± 0.6
Field (natural)	Neutral soil	2	6.5	5.2 ± 0.2
	Acidic soil	0	4.1	54.7 ± 0.4
	Acidic soil	2.2	4.4	48.9 ± 0.7
	Acidic soil	6.2	5.3	13.5 ± 0.1

### 3.4. Protein Sequence and Phylogenetic Analyses for the Identification of GmALS3 Orthologs in Other Crops

To analyze the relationship between orthologous proteins from various plant species, a homology search of GmALS3 was carried out using BLASTP. The phylogeny tree contains ≥75% sequence identity with GmALS3 (Figure 4). AtALS3 was grouped with NtALS3, while GmALS3 (Glyma.10G047100) and Glyma.03G175800 were grouped with putative ALS3 proteins from other legumes. This suggests that *G. max* has two AtALS3 orthologs, but our wet lab studies showed that only *Glyma.10G047100* was responsive to Al stress. On the other hand, OsSTAR2 was grouped with TaALS3 and ZmALS3. These findings show that the genes for ALS3 proteins are conserved in a wide range of plant species.

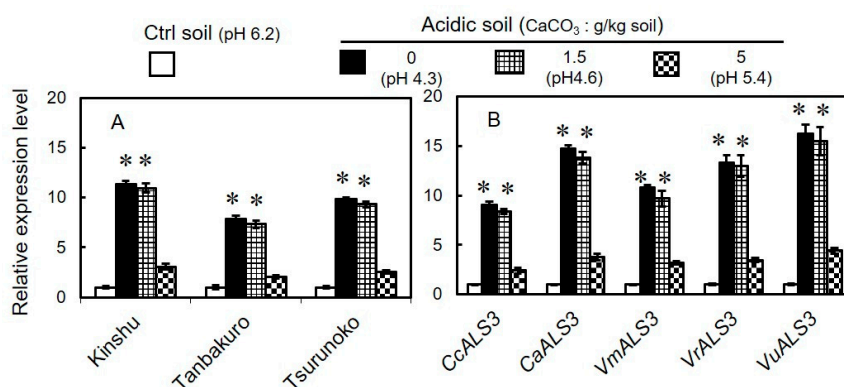


**Figure 4.** Protein sequences having ≥75% sequence identity with GmALS3. A phylogenetic tree was generated using Phylogeny.fr. Branch-support values were calculated using the bootstrapping procedure indicated in red. The tree was constructed from a sequence alignment of the following proteins: *Arabidopsis thaliana* AtALS3 (GenBank: OAP08021), *Nicotiana tabacum* NtALS3 (GenBank:

107801961), *Oryza sativa* OsSTAR2 (ALS3) (GenBank: 4337635), *Triticum aestivum* TaALS3 (Phytozome: Tres\_1DS\_8CA54B2AF), *Glycine max* Glyma.10G047100 (Phytozome: Glyma.10G047100), *Glycine max* Glyma.03G175800 (Phytozome: Glyma.03G175800), *Cicer arietinum* CaALS3 (Phytozome: Ca\_11996), *Medicago truncatula* MtALS3 (GenBank: 11431871), *Cajanus cajan* CcALS3 (GenBank: AWI62593), *Phaseolus vulgaris* PvALS3 (Phytozome: Phvul.001G172900), *Vigna angularis* VaALS3 (GenBank: 108328314), *Vigna mungo* VmALS3 (GenBank: ALX81167), *Vigna radiata* VrALS3 (GenBank: 106757328), *Zea mays* ZmALS3 (Phytozome: GRMZM2G053991) and *Vigna unguiculata* VuALS3 (Phytozome: Vignun01g155100). \* Indicates that the proteins have previously been reported as having Al tolerance regulated by STOP1 under Al stress, † Indicates that the proteins were previously reported to be inducible under Al stress.

### 3.5. Expression of GmALS3 in Different Varieties of Soybeans and Its Orthologs in Other Legumes Grown in Acidic Soils

We analyzed the expression of *GmALS3* in the shoots of three different varieties of soybeans (Kinshu, Tanbakuro and Tsurunoko) grown in artificial acidic soil with different levels of  $\text{CaCO}_3$  (same conditions as in Figure 3E). Relative expression levels were slightly different among varieties, but all three varieties showed suppression of *GmALS3* at high levels of  $\text{CaCO}_3$  (Figure 5A). These results suggest that the expression of *GmALS3* is a useful biomarker that is robust and independent among soybean genotypes.



**Figure 5.** Expression level of *GmALS3* in soybean varieties and its orthologs in other legumes grown in Al-toxic and nontoxic soils. (A) *GmALS3* expression levels were quantified in the shoots of three soybean genotypes (Kinshu, Tanbakuro and Tsurunoko) grown in artificial soils; (B) expression level of *GmALS3* orthologs in the shoot of various legumes (*Cajanus cajan*, *Cicer arietinum*, *Vigna mungo*, *Vigna radiata* and *Vigna unguiculata*) grown in artificial soils. Plants were grown for two weeks in neutral soil (addition of  $\text{CaCO}_3$  2 g/kg soil) as a control and in acidic soil with addition of different levels of  $\text{CaCO}_3$  (0, 1.5 and 5 g/kg soil) as a treatment. Expression level quantified by qRT-PCR. *GmUBQ10* and *Actin* (for other legumes) were used as an internal standard. Data given as mean and  $\pm$  SE ( $n = 3$ ). Asterisks indicate significant differences (Student's *t*-test,  $p < 0.05$ ) compared to control soil.

To determine whether *GmALS3* orthologs show similar Al-dependent expression in other legumes, we identified the orthologs and quantified their expression levels in five other legumes (*C. cajan*, *C. arietinum*, *V. mungo*, *V. radiata* and *V. unguiculata*). A BLASTP search of *GmALS3* identified a single copy of the orthologue in the genome of each species (Figure 4). Expression of these orthologs was significantly induced in the shoots of each respective legume grown in acidic soils (Figure 5B). These expression levels were suppressed with high levels of application of  $\text{CaCO}_3$  (Figure 5B). These results suggest that orthologs of *GmALS3* could be utilized to evaluate Al toxicity in other legumes.

#### 4. Discussion

Recently, a number of transcriptional biomarkers have been identified in crop plants. For example, Yang et al. [22] identified a set of specific N-responsive transcriptional biomarkers in different maize genotypes grown under various N conditions, whereas Zhao et al. [49] reported a transcriptional biomarker (*miR399*) in citrus that can be used for managing HLB-positive (Huanglongbing; Citrus greening disease) plants suffering P starvation. In the present study, we found that an orthologue of *ALS3* [32], *Glyma.10G047100* (*GmALS3*; Table 1), is inducible in the shoots of soybeans in response to Al treatments applied to the roots (Figure 1). The expression of *GmALS3* was observed to be almost specific to rhizotoxic Al treatment (Figure 2). In addition, *GmALS3* expression was induced under acidic soil culture conditions and was suppressed by liming (Figure 3). Although direct transcript analyses would be expensive to adopt for agronomic purposes, our findings indicate that *GmALS3* expression could be used as a quantitative measure of the response of soybeans to different levels of lime in acidic soils (Figure 3).

Although we detected two orthologs of *AtALS3* in the soybean genome based on BLASTP searches (Table 1), we found that only *GmALS3* showed Al-dependent expression in the shoot (Figure 1). The expression profiles of shoot *GmALS3* in response to rhizotoxic ions (indicating a nearly specific response to Al; Figure 2) are similar to those of *ALS3* in *Arabidopsis* and tobacco [33]. In that study, we also found that several other genes [*Hydrolase* (Class I glutamine amidotransferase-like superfamily protein), *PGIP1* (POLYGALACTURONASE INHIBITING PROTEIN 1) and *GolS3* (GALACTINOL SYNTHASE 3)] were also specifically induced by Al in the shoots of *Arabidopsis*. However, expressions of the orthologs of these genes were not specific to Al in soybeans (Figure 2). These results indicate that unlike other Al-responsive genes, the Al-specific expression of *ALS3* orthologs is conserved, which may be attributable to the conservation of STOP1 and *ALS3* in different plant species. The STOP1-regulated Al-inducible expression of *ALS3* has been experimentally validated in *Arabidopsis* [26], tobacco [50], Eucalyptus [51] and rice [27]. In addition, *ALS3* expression in the shoots has previously been demonstrated to be specific to Al and regulated by STOP1 in *Arabidopsis* and tobacco [33].

Several genes regulated by STOP1 have been found to be upregulated in response to other exogenous factors, including low pH and P deficiency, which are typically co-excisiting stressors in acidic soils [52]. For example, the expression of *AtALMT1*, one of the major Al-tolerance genes regulated by STOP1 [53], is induced by P deficiency [54] and low pH [40]. In contrast, however, it has been reported that the expression of *ALS3* in *Arabidopsis* and rice is not induced in response to low pH or other co-excisiting stressors in acidic soils [55,56]. These observations accordingly indicate that unlike other Al-tolerance genes, the expression of *ALS3* is specifically induced by Al. Furthermore, on the basis of our analysis of *GmALS3* in the shoots of soybeans exposed to different pH and deficiencies in different macronutrients (N, P and K), we found that the expression of *GmALS3* was affected neither by pH nor by macronutrient deficiency (Figure 2 and Figure S2), indicating that *GmALS3* expression is specific to Al and could be used to evaluate Al rhizotoxicity in soybeans.

When soybeans were grown in acidic soil treated with levels of liming that have been shown to eliminate soil  $\text{Al}^{3+}$  toxicity, we observed that the shoots showed a reduction in Al accumulation (Figure 3), which was correlated with the expression of *GmALS3* and equivalent to that in the shoots of soybeans grown under control conditions (unstressed) (Figure 3). We also found that the growth and biomass of these plants were similar to those of the control plants (unstressed) (Figure 3, Figures S3 and S4; Table S3). These results clearly indicate that expression of *GmALS3* in the shoots can be used as a biomarker to evaluate the ameliorative effects of lime in acidic soil. In addition, we found the expression of *GmALS3* in the shoots to be robust among three genotypes of soybeans (Kinshu, Tanbakuro and Tsurunoko) grown in acidic soil (Figure 5).

We also identified genes having the highest homology with *GmALS3* ( $\geq 75\%$  sequence identity) in various legumes (Figure 4) and quantified the expression of these genes in the shoots of plants grown in acidic soils amended with levels of lime sufficient to neutralize soil acidity. Based on this analysis, we found that the expression of *GmALS3* orthologs in different legumes was correlated with the level

of liming in acidic soil (Figure 5). These results indicate that the expression of *GmALS3* could be used to monitor the levels of soil Al toxicity in crops (at least in legumes) grown in acidic soils.

## 5. Conclusions

We found that *GmALS3* (*Glyma.10G047100*) was specifically upregulated by Al in the shoot when exposed to various rhizotoxic stress treatments. In addition, *GmALS3* expression was correlated with the amount of exchangeable Al in acidic soils. These results indicate that *GmALS3* expression, which has been validated to be Al stress-specific, robust and highly quantitative among soybean genotypes, is generally applicable for managing soybean production in acidic soils. The demand for this type of sensitive biomarker for additional agronomic traits is widespread, and the results reported here provide a model for their continued development in crops.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2073-4395/10/9/1228/s1>, Figure S1: Semiquantitative PCR expression of internal standards used in this study to normalize the gene of interest in qRT-PCR. Figure S2: Rhizotoxic ions effects on root growth and *GmALS3* expression in soybeans. Figure S3: Soybean shoots growth inhibition in neutral and acidic soil. Figure S4: Soybean roots growth inhibition in neutral and acidic soil. Figure S5: Al content of the roots of soybean plants grown in neutral and acidic soil. Table S1: Sequence information of primers used for qRT-PCR. Table S2: Efficiency of designed primer pairs used for qRT-PCR amplification. Table S3: Changes in shoots, fresh and dry weight of soybeans grown in artificial and natural soils. Table S4: Changes in roots, fresh and dry weight of soybeans grown in artificial and natural soils.

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