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ORIGINAL ARTICLE

Quantitative Gene Expression Analysis Across Contrasting Linseed Genotypes Identifies New Candidates for Aluminum Toxicity and Low-Phosphorus Tolerance

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ABSTRACT

In the North East Hill Region (NEHR) of India, acidic soils cause aluminum (Al^{3+}) toxicity and phosphorus (Pi) deficiency, severely limiting crop productivity. Linseed offers potential for enhancing cropping intensity and diversification in this region. Contrasting oilseed-type linseed genotypes identified for yield traits under acidic soils were further validated for root and shoot traits through hydroponic screening. Using an orthology-based approach, eight gene families—*MATE*, *ALMT1*, *MGT1*, *PT6*, *PAP15*, *WD40-38*, *SPL4*, and *ACA3*—known to confer Al^{3+} toxicity and P deficiency tolerance in other plant species were targeted. Thirty-one genic regions were identified. In roots of four genotypes (Lus 37, Lus 237, Lus 4, and Lus 49) exposed to Al^{3+} toxicity, *UGT74*, *MATE1365*, *MGT1272*, and *MGT1577* were upregulated in tolerant genotypes, indicating their probable roles in detoxification, transport, and ion homeostasis. Under low Pi conditions, eleven genes exhibited differential expression in shoots and roots of Lus 37, Lus 29, and Lus 4, with *PT6826* and *PT6635* upregulated in roots of tolerant Lus 37. Overall, *LusMATE1365*, *LusUGT74*, and *LusPT6635* were identified as key candidate genes that could potentially confer tolerance to Al^{3+} toxicity and P deficiency. These insights will aid molecular breeding for linseed adaptation to acidic soils of NEHR.

1 | Introduction

Acidic soil affects 49 million hectares of India's total land area, with 24 million hectares having a pH below 5.5 (Kumar et al. 2012). Acidic soils are predominantly distributed in the north-eastern hill region (NEHR) of India, where nearly 65% of the cultivated area is affected by severe soil acidity (pH < 5.5). Productivity potential of acidic soils is estimated to range from 25% to 80% less than that of neutral soil, primarily due to aluminum (Al^{3+}) toxicity and phosphorus (Pi) deficiency in the soil, both of which lead to poor growth and yield reduction. One of the major problems associated with soil acidity is phosphorus fixation (Singh et al. 2011; Tyagi and Rai 2012; Das et al. 2017). Additionally, in soils with a pH below 5, soluble Al^{3+} ions inhibit root growth (Zheng 2010; Liu et al. 2022).

In response to Al toxicity, plants have evolved different adaptation strategies including detoxification, modification of cell wall properties, transport of Al to shoots and Al storage in innocuous forms (Sade et al. 2016). One of the best-characterized mechanisms is organic (such as malate, citrate) anion exudation which chelates Al^{3+} and prevents its entry into the root (Yang et al. 2013). This is achieved by the coordinated effort of membrane transporters and regulatory components located in the root cells. Once in the rhizosphere, organic

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anions form stable complexes with Al, thereby conferring Al toxicity tolerance (Sasaki et al. 2006).

Transporters mediating organic anion efflux predominantly belong to the aluminum-activated malate transporter (ALMT) and multidrug and toxic compound efflux (MATE) families, whose roles in Al^{3+} toxicity tolerance have been demonstrated across several plant species, including cereals and model systems, through malate- and citrate-mediated chelation of Al^{3+} in the rhizosphere, thereby limiting its entry into root tissues (Sasaki et al. 2006; Yang et al. 2013; Zhou et al. 2013). Variation in expression patterns, allelic composition, and regulatory control of *ALMT* and *MATE* genes has been shown to significantly influence Al^{3+} toxicity tolerance levels among genotypes (Ma et al. 2002; Maron et al. 2010; Maron et al. 2013; Arenhart et al. 2014; Tyagi et al. 2020). Collectively, these studies highlight the conserved nature of Al^{3+} detoxification mechanisms while emphasizing the importance of species- and genotype-specific regulation in shaping adaptive responses to acidic soil conditions.

Phosphorus (Pi) deficiency is one of the major constraints in acidic soil, which can severely limit the yield of plants. Normally, P is taken up by plants as HPO_4^{2-} and $H_2PO_4^-$ but in acidic soils, P is fixed by metal oxides. The transport and regulation of phosphate ions are carried out by various signal molecules, including transporters, transcription factors, hormones, etc (Gu et al. 2016; Tyagi et al. 2021).

Genotype-specific variation in Pi deficiency responses has been reported across crops, indicating a strong genetic component underlying adaptation to low P environments. *Phosphate Transporters* (*PT*) play a significant role in enhancing plant tolerance to low-phosphorus (P) conditions across various species like barley, soybean, foxtail, etc by promoting phosphate acquisition efficiency (Zhang et al. 2016; Roch et al. 2020; An et al. 2023). Transcription factors like MYB, WRKY, *SPL4*, *WD40* and purple acid phosphatases (*PAPs*) help release phosphate from organic compounds in the soil, aiding in phosphate mobilization.

Linseed (*Linum usitatissimum*) is valued for its high omega-3 fatty acid content, dietary fiber, and protein, making it a recognized superfood. The crop is also important industrially, with its fiber used for linen production and oil used in paint industry. Recently, there has been an increase in linseed cultivation because of its wide industrial (fiber and oil) and nutraceutical applications (high omega-3 fatty acid and anti-cancer properties due to high amount of lignans) (Goyal et al. 2014; Kaur et al. 2023). Pi deficiency and Al^{3+} toxicity in acid soils pose a challenge for productivity of linseed (Bezil et al. 2025). Therefore, identification of varieties tolerant to both Pi deficiency and Al^{3+} toxicity is needed to reduce liming and fertilizer requirements for yield improvement. However, very little is known about the mechanism of linseed tolerance to Pi deficiency and Al^{3+} toxicity under acidic soil conditions.

In the present work, we validated contrasting genotypes identified for yield traits under acidic soils for shoot and root traits using hydroponic screening. Using an orthology-based approach, 31 genic regions underlying eight gene families known to confer Al^{3+} toxicity and Pi deficiency tolerance in other plant species were identified and targeted for expression analysis. Overall, this led to the identification of genotypes and key candidate genes that could potentially confer tolerance to Al^{3+} toxicity and Pi deficiency. These insights will aid molecular breeding for linseed adaptation to acidic soils of NEHR.

2 | Materials and Methods

2.1 | Plant Material and Hydroponics

A total of 204 linseed germplasm accessions obtained from the Germplasm Evaluation Division, ICAR-NBPGR, IARI Campus, were evaluated under the acidic upland field condition for two seasons (unpublished data). Based on the yield contributing traits, a set of six contrasting genotypes were identified (Supporting Information Table S1). These six genotypes were validated by screening under Pi deficiency and aluminum (Al^{3+}) toxicity stress conditions using a modified Hoagland solution (Hoagland 1920). Briefly, 50 seeds of each linseed genotype were soaked in distilled water for 1 day and transferred to moist germination paper for 5 days at 22°C. After 5 days, seedlings were transferred to cups containing modified Hoagland solution. Each cup contained five plants, and three replications for each genotype were done. After 7 days, plants were screened separately for Al toxicity or phosphorus deficiency tolerance. The solution was changed every 2 days. Seedlings were grown under controlled hydroponic conditions and subjected to Al^{3+} toxicity to evaluate phenotypic responses. For phenotypic screening, a concentration of 150 μM $AlCl_3{\cdot}6H_2O$ was applied for 10 days, which was selected based on preliminary optimization and published studies demonstrating reliable discrimination between Al^{3+}-toxicity tolerance and sensitive genotypes without causing irreversible damage. This concentration primarily reflects chronic Al^{3+} toxicity relevant to root growth inhibition under acidic soil conditions. After 10 days, data for traits like root length (cm), root fresh weight (g), and shoot fresh weight (g) were recorded, along with the degree of haematoxylin staining were recorded.

Similarly, for phosphorus deficiency tolerance screening, plants were divided into two sets and transferred to cups containing Hoagland solution either 0.5 mM P (control) or 0.025 mM P (treatment). After 14 days, root length (cm), root fresh weight (g) and shoot fresh weight (g) were recorded. Derived parameters such as relative root length (RRL), relative root fresh weight (RRFW), relative shoot fresh weight (RSFW), relative root dry weight (RRDW), relative shoot dry weight (RSDW), and root biomass: shoot biomass ratio were also computed. Pi content in the leaves of seedlings was determined by the phosphovanadate method (Hanson 1950) after digestion in a mixture of HNO_3 and $HClO_4$ (3:1).

2.2 | Haematoxylin Staining

Ten-day-old seedlings grown in hydroponics under the control or treatment of Al stress condition, root samples were used for haematoxylin staining (0.2% haematoxylin (Himedia) and 0.02% potassium iodide) following the standard procedure (Tyagi et al. 2020). After staining, the roots were visually evaluated by three independent referees and scored for staining intensity on a 0-to-5 scale. The average of three scores was used for analysis.

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2.3 | Orthologs and Candidate Gene Identification, and Primer Designing

Genomes of several linseed genotypes (both fiber- and oil-seed type) from Canada (CDC Bethune), China (Longya-10, Hieya-14 and Yiya-5), and Russia (Atlant) are publicly available. Transcriptome data for fiber-type linseed are also accessible, along with validated primers for expression analysis (Huis et al. 2010). Many genes responsible for imparting Al^{3+} toxicity and Pi deficiency tolerance have been identified in other crops; however, only a limited number are reported in linseed. We used information available from 3 species, rice, maize, and Arabidopsis to identify functionally validated transporters and transcription factors with phenotypic information available. Using the available gene sequences found in Genebank database, the query sequence (protein and nucleotide) was retrieved from the National Centre for Biotechnology Information (NCBI) and used as input for sequence for tBLASTx searches, restricting the output to *Linum usitatisimum* (taxid:4006) (Supporting Information Figure S1).

Since linseed is distantly related to rice, maize, and wheat, sequence similarity ranging from very high to very low was observed. The query sequences with high similarity and lowest e-value were selected as putative orthologs. The protein sequences of the selected putative genes were retrieved from NCBI and BLAST searched in the Phytozome database against the *Linum usitatisimum v1.0* (v13; https://phytozome-next.jgi.doe.gov/) (Goodstein et al. 2012). The orthologs with a higher percentage identity and the lowest e-value were selected. Additionally, the presence of conserved domains in the selected orthologs was also verified using the UNIPROT and Phytozome database. The transcript details of the selected orthologs, including locus ID, chromosomal location, and percentage identity, were also retrieved and expression profiles were checked in the FIBexDB (Plant Fiber Expression database, https://ssl.cres-t.org/fibex/) (Supporting Information Figure S1).

For the newly identified candidate genes, primers were designed using the NCBI primer BLAST tool, with an annealing temperature of 60°C, and a product length of 75-226 bp was fixed as the criterion for the design of primers. Primers showing minimal off-target hits in Linum (taxid:4006) were selected and synthesized by Eurofins Genomics India Pvt Ltd., India. The lyophilized primers were dissolved in 1X TE buffer to obtain 100 μM stock and 10 μM working solutions.

2.4 | Growth Conditions, RNA Isolation and cDNA Synthesis

Fifty seeds for each genotype were germinated on water-soaked filter paper at 22°C for 5 days. For the P-deficiency stress, three genotypes, i.e. Lus 4 (IC0320983), Lus 29 (IC0498576), and Lus 37 (IC0096500) were used under the same hydroponic conditions discussed above. On the 14th day after treatment, shoot and root tissues from control and treated plants were harvested separately, flash-frozen in liquid nitrogen and stored for RNA isolation at −80°C freezer.

For Al toxicity, four genotypes, i.e., Lus 4 (IC0320983), Lus 29 (IC0498576), Lus 37 (IC0096500), and Lus 237 (IC0498381) were used under growth conditions similar to those in Dmitriev et al. 2016b. Briefly, uniform seedlings were transferred to Petri plates containing filter paper soaked in 0.5 mM $CaCl_2$ (pH 4.5) and kept for 24 h for preconditioning the effects of Al^{3+}. Seedlings were exposed to 500 μM $AlCl_3{\cdot}6H_2O$ for 4 h (T4) and 12 h (T12) to capture early transcriptional responses. The use of a higher Al^{3+} concentration and shorter exposure duration for qRT-PCR analysis was intentional and distinct from phenotyping experiments, as short-term high-dose treatments are commonly employed to induce measurable transcriptional changes without confounding long-term growth effects.

Total RNA was extracted using Trizin RNA extraction reagent (GCC Biotech, India). cDNA was synthesized using SuperScript III First-Strand Synthesis system (Invitrogen, USA) following the manufacturer's protocol. Briefly, 2 μg of total RNA was reverse transcribed with 50 μM oligo dT and 10 mM dNTP mix, and reactions were incubated at 50°C for 50 min, followed by enzyme inactivation at 85°C for 5 min.

2.5 | Expression Analysis and Validation Using qPCR

The reverse transcriptase primers (RT) were standardized on genomic DNA of the genotype Lus 29. Gradient PCR with annealing temperatures of 56°C–60°C and 30 cycles were performed to determine optimal annealing temperature, with 56°C giving clear amplicons of expected size for most genes. Linseed *EF1A* (*Elongation Factor 1A*) was used as a reference gene (Huis et al. 2010). The qRT-PCR was performed using a CFX96 Real-Time System (Bio-Rad), and specificity was verified by melt curve analysis. Three biological and three technical replicates of each sample were analyzed. Expression values were normalized with *EF1A* transcript level, and relative expression calculated as reported previously (Bhutia et al. 2021). Correlations between the available transcriptome data for fiber-type in the database and qPCR expression for oil-seed type were evaluated using the Pearson Correlation Coefficient.

2.6 | Statistical Analysis

All experiments were conducted using at least three independent biological replicates. Phenotypic data were analyzed using Student's *t*-test to compare control and treated plants, and results are presented as mean ± standard deviation (SD). For qRT-PCR, relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with *EF1A* as the internal reference gene. Statistical significance was assessed using one-way ANOVA followed by appropriate post-hoc comparisons. Differences were considered statistically significant at $p < 0.05$, unless otherwise stated.

3 | Results

3.1 | Al Toxicity and Phosphorus Deficiency Screening Under Hydroponic Conditions

Upon evaluation of 204 diverse linseed materials (unpublished data) under acidic lowland and upland field conditions, six

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representative genotypes (Lus 4, Lus 29, Lus 37, Lus 38, Lus 49, and Lus 237; Supporting Information Table S1) contrasting for yield contributing traits were selected for seedling stage phenotypic screening under Al toxicity and low Pi conditions separately.

3.2 | Al Toxicity Screening

A significant reduction in root length was observed in Al-treated samples of Lus 4, Lus 29, and Lus 49 (Figure 1). Treated samples of Lus 37 and Lus 38 showed higher root length than the control, though the difference was not significant (Figure 1). Differences in the root fresh weights among the six genotypes were also observed in Al toxicity, but not always significant (Lus 4, Lus 29, and Lus 237). However, notable differences between the control and treated conditions were observed in Lus 37, Lus 38, and Lus 49. Significant differences in shoot fresh weight between the control and treated conditions were observed in genotypes Lus 37 and Lus 38.

Haematoxylin staining (Figure 2) with RRL (relative root length) data showed that root growth inhibition and Al^{3+} accumulation in roots are correlated. Lowest RRL values were recorded in Lus 4 and Lus 49, showing intense staining, while Lus 37 and Lus 237 had faint stains and less inhibition.

3.3 | Low P Tolerance Screening

A significant reduction in root length was observed in Lus 4, Lus 29, Lus 49, and Lus 237 under Pi deficit conditions (Figure 3). Root fresh weights also declined in Lus 4, Lus 29, Lus 38, Lus 49, and Lus 237 compared to controls.

3.4 | Identification of Contrasting Genotypes for Expression Studies

Tolerant and susceptible genotypes were identified based on hydroponics screening traits such as RRL, RRFW, and RSFW (Supporting Information Table S1).

Genotypes Lus 4 and Lus 29 showed greater reduction in root growth compared with Lus 37 and Lus 38. Similarly, root fresh weight and shoot fresh weight of Lus 4 and Lus 29 were less compared with Lus 37 and Lus 38 between the control (0.5 mM Pi) and treatment (0.025 mM Pi). Hence, Lus 4 and Lus 29 were considered as susceptible genotypes and Lus 37 as tolerant genotypes under Pi deficiency.

Similarly, phenotypic traits along with haematoxylin staining were used for Al^{3+} toxicity tolerance assessment. Under hydroponics screening, root growth inhibition was observed in high Al^{3+} exposure at low pH 4.5 (Krasnov et al. 2019). Both the tolerant and susceptible genotypes showed a varied degree of changes in the recorded phenotypic traits. Reduction in the root length was more pronounced in the susceptible genotypes (Lus 4, Lus 29, and Lus 49), with more haematoxylin staining. While in Lus 237, less reduction in root length with faint stain was observed and in Lus 37 and Lus 38, the root length was more compared to the control condition with faint stain. Hence, Lus 4 and Lus 49 were considered as susceptible genotypes and Lus 37 and Lus 237 as tolerant genotypes under Al^{3+} toxicity.

Correlation analysis for Al^{3+} toxicity screening revealed that RSDW showed positive correlation with RRDW ($r = 0.85$) and RLRN ($r = 0.84$) at 5% level of significance. RRL also positively correlated with SY ($r = 0.93$) at 1% level of significance. RRDW showed negatively correlation with haematoxylin staining ($r = 0.95$) at 5% level of significance.

Correlation among the traits for low Pi screening revealed that RRDW showed positive correlation with RRFW ($r = 0.97$) at 0.1% level of significance; with RSDW ($r = 0.93$) at 1% level of

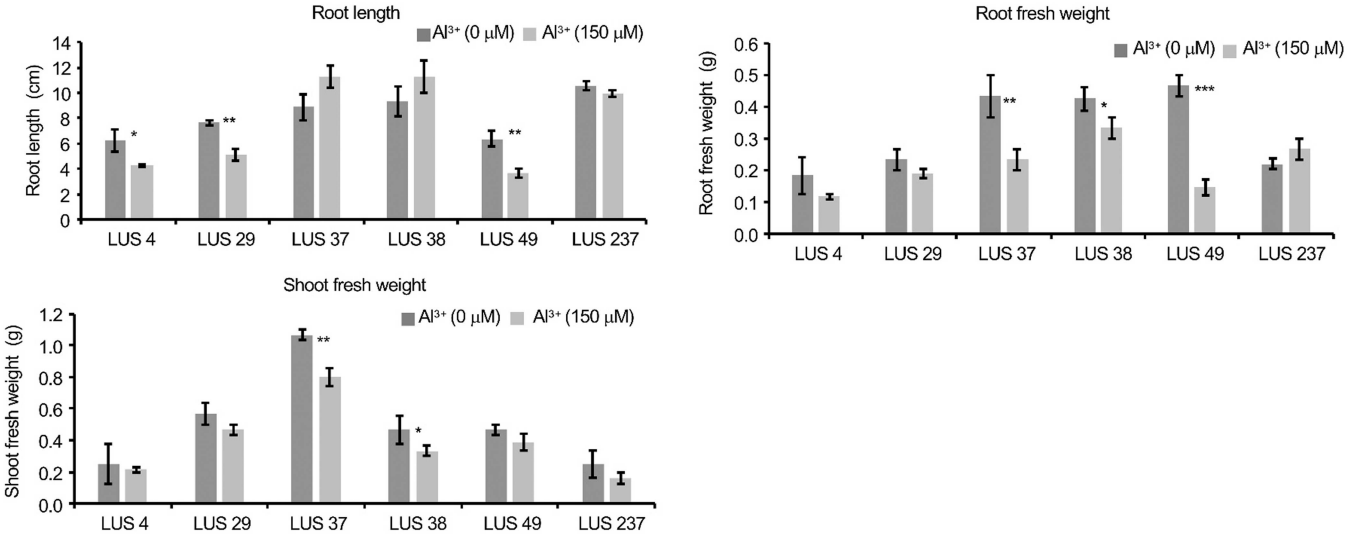


FIGURE 1 | Root and shoot growth responses of linseed genotypes under aluminum toxicity. Root length, root fresh weight, and shoot fresh weight of 5-day-old seedlings exposed to Al toxicity for 10 days under hydroponic conditions. Values represent means ($n = 15$) ± standard error. The X-axis indicates the genotypes, and the Y-axis indicates root length (cm), root fresh weight (g) and shoot fresh weight (g). The bar graphs depict phenotypic responses under control (Al^{3+} 0 μM) and treatment (Al^{3+} 150 μM) conditions. Statistical significance between control and treatment was determined by student's *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Genotypes: Lus 4 = IC0320983; Lus 29 = IC0498576; Lus 37 = IC0096500; Lus 38 = IC0345423; Lus 49 = IC0499100; and Lus 237 = IC0498381.

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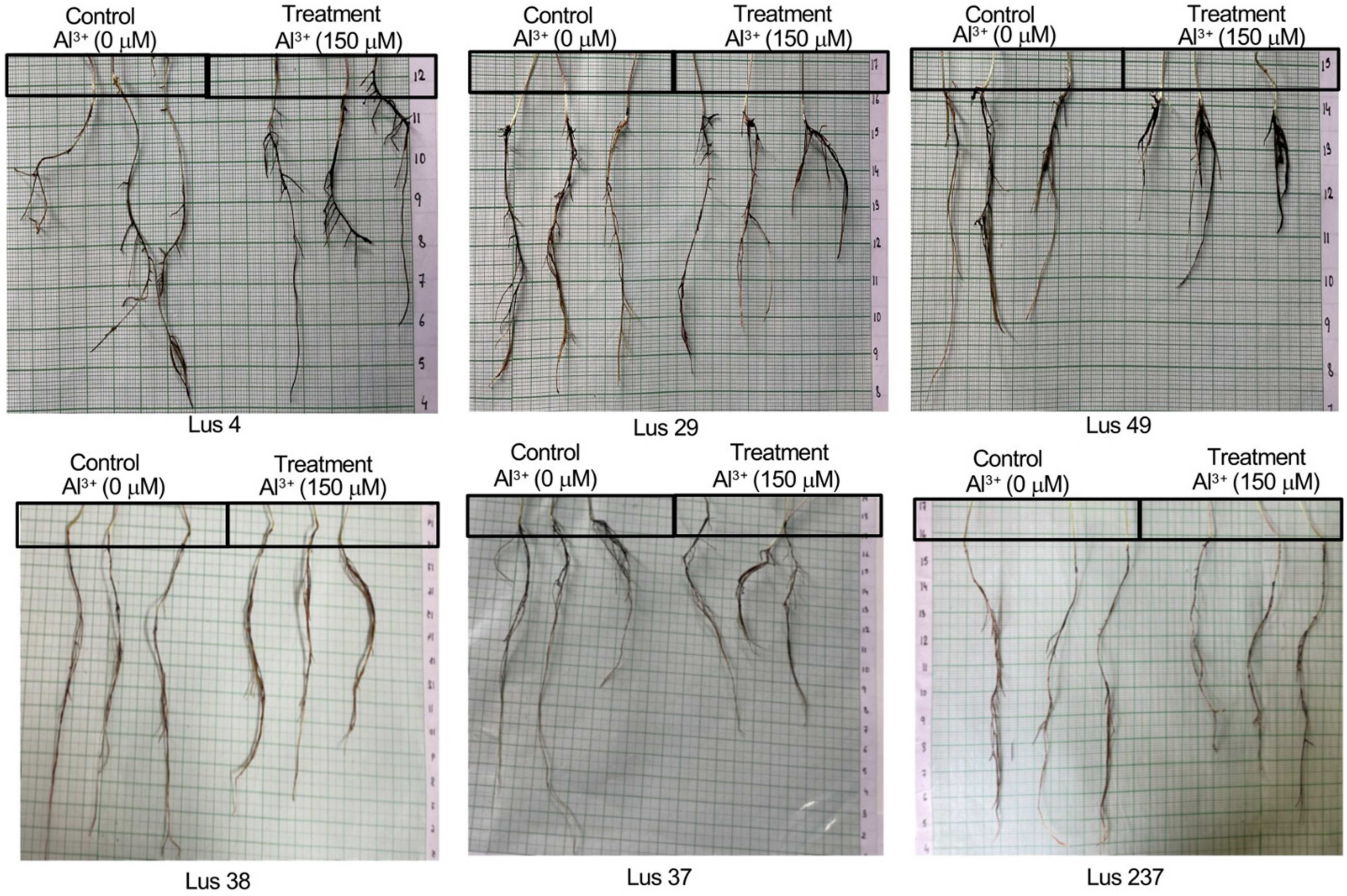


FIGURE 2 | Haematoxylin staining reveals aluminum accumulation patterns in linseed roots. Haematoxylin staining of 5-day-old seedlings grows in modified Hoagland solution under control (Al^{3+} 0 μM) and treatment (Al^{3+} 150 μM) conditions for 10 days. One plant each from each of the three replications from both control and treatment conditions was used for hematoxylin staining. Images were captured, and each large square (thick border) containing 10 small squares represents 1 cm^2. The genotypes included were Lus 4 = IC0320983; Lus 37 = IC0096500; Lus 49 = IC0499100; and Lus 237 = IC0498381.

significance, and with RSFW ($r = 0.91$) and RSL ($r = 0.81$) at 5% level of significance. RRFW also showed positive correlation with RSDW ($r = 0.93$) and RSFW ($r = 0.86$) at 1% and 5% levels of significance, respectively. RSL showed correlation with RRL ($r = 0.86$), RSDW ($r = 0.87$), and RRDW ($r = 0.81$) at 5% level of significance. RSDW showed correlation with RSFW ($r = 0.81$) at 5% level of significance.

3.5 | In-Silico Identification of Putative Gene Orthologs in Linseed

Using an orthology-based approach, a set of eight gene families (transporters - *MATE*, *ALMT1*, *MGT1* and *PT6*; *PAP15*; transcription factors - *WD40-38*, *SPL4* and *ACA3*) known to confer tolerance to Al^{3+} toxicity and low Pi in other species were targeted to understand molecular mechanisms of this trait in linseed (Supporting Information Table S2).

A total of 11 genic regions were identified for Al^{3+} toxicity-responsive genes which include five multidrug and toxic compound extrusion (*MATE*) transporters, two aluminum activated malate transporter 1 (*ALMT*) transporters, and four magnesium transporter 1 (*MGT1*) (Supporting Information Table S3). For the MATE transporter, out of 21 orthologs identified across *Linum* genomes, five were selected, showing low e-values and 56%–60% sequence similarity. The two highly conserved functional domains, PF01554, which encodes the multi-antimicrobial extrusion (MATE) protein, was observed in all five selected orthologs. Additionally, the PF13456, ribonuclease H domain, was also found in Lus10029517 (Table 1). Similarly, two *ALMT* orthologs were selected with 48%–49% identity. These two orthologs also had the lowest e-value and has a conserved ALMT domain (PF11744). Similarly, the *OsMGT1* transporter gene had 14 orthologs, out of which four were selected with low e-values, and sequence identity ranging from 39% to 45%. These orthologs have a conserved (PF1544) CorA-like Mg^{2+} transporter domain.

For Pi deficiency-responsive genes, twenty genic regions were identified, including six *PT6*, five *PAP15*, two *WD40*-38, two *SPL4*, and five *ACA3* genes. Five purple acid phosphatase (*PAP15*) orthologs out of 40 were selected with 60%–73% sequence similarity (e-value = 0), having three conserved domains (PF16656, PF00149, PF14008) similar to *AtPAP15*. *OsPT6*, a phosphate transporter gene in rice, had a total of 20 orthologs in linseed. Six were selected based on the lowest e-value, high identity (69%–75%) with two conserved domains (PF00083 and PF07690) (Table 1). The orthologs of Lus10022548 and Lus10024884 have an additional sugar

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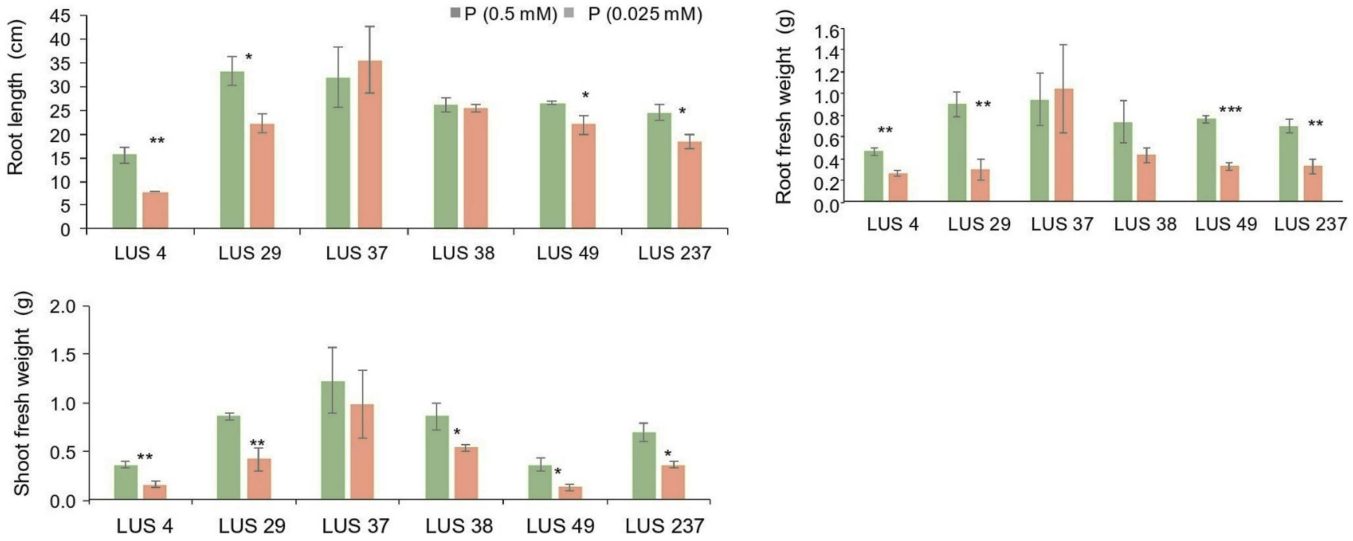


FIGURE 3 | Root and shoot growth responses of linseed genotypes under phosphorus deficiency. Root length, root fresh weight and shoot fresh weight of 5-day-old seedlings screened under phosphorus-deficient conditions for 15 days. Values plotted are means ($n = 15$) and bars indicate ± standard errors. The X-axis indicates the list of genotypes and Y-axis shows the root length (cm), root fresh weight (g) and shoot fresh weight (g), respectively, under hydroponic conditions. The bar graph represents average phenotypic values under control P (green; 0.5 mM) and treatment P (red; 0.025 mM) conditions. Statistically significant differences between the control and treatment were determined by the student's *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Lus 4 = IC0320983; Lus 29 = IC0498576; Lus 37 = IC0096500; Lus 38 = IC0345423; Lus 49 = IC0499100; and Lus 237 = IC0498381. [Color figure can be viewed at wileyonlinelibrary.com]

transporter domain, PF00083, present in *OsPT6*. *OsSPL4*, a squamosa promoter-binding-like transcription factor from rice, has 32 orthologs. Two had the lowest e-value, 67% identity with a conserved domain, PF03110. *OsWD40-38*, a WD40 repeat-containing protein in rice, has 142 orthologs in linseed. Among them, only 2 were selected (e-value = 0, 81% identity), and they have the same conserved domain, PF00400, found in rice. *OsACA3*, type II Ca^{2+} ATPase gene has 89 orthologs, out of which five were selected based on 0 e-value, 67%–72% sequence similarity and five conserved domains PF00689, PF00122, PF13246, PF00690, and PF12515 (Table 1: Supporting Information Table S3). In all the orthologs, viz. Lus10011522, Lus10019300, Lus10007736, Lus10025199, and Lus10018687 all five domains were present. PF12515 was lacking in Lus10025199. Another conserved domain, PF00702, same is present in rice phosphate transporters, was also present in the linseed ortholog Lus10018687. Thus, a total of 31 genic regions were identified in linseed for Al^{3+} toxicity and Pi deficiency responsive genes (Supporting Information Table S3) and targeted for qRT-PCR (Supporting Information Table S4).

3.6 | Expression Study of Genes Reported for Imparting Al^{3+} Toxicity Tolerance

Expression profiling of linseed orthologs associated with Al^{3+} toxicity tolerance was performed using root tissues from four genotypes subjected to 4 h (T4) and 12 h (T12) Al^{3+} toxicity. *LusMATE1365* showed no expression in Lus 37 roots, while Lus 237 exhibited 17-fold upregulation after 12 h treatment but no expression at 4 h. Lus 4 expressed *LusMATE1365* only under control conditions, and Lus 49 showed similar expression in control and 12 h samples but none at 4 h (Figure 4a). *LusMGT1577* was expressed only in T12 samples of Lus 37. In Lus 237, expression was consistent under control and T4 but absent at T12. In Lus 4, control and T12 showed equal expression, while Lus 49 showed no expression at T4 (Figure 4b). *LusMGT1272* exhibited slight upregulation in Lus 37, downregulation in Lus 237, and no expression in Lus 4 at 12 h. Lus 49 showed similar expression across all treatments (Figure 4c). *LusGSTU8* showed consistent expression across treatments in Lus 37, Lus 4, and Lus 49, but moderate downregulation at T12 in Lus 237 (Figure 4e). No differential expression was observed for *LusGST23*.2 and *LusUGT71* across genotypes (Figure 4d,f). *LusUGT74* showed no change in Lus 37; in Lus 237, it was slightly downregulated at 12 h and undetectable at 4 h. In Lus 4, basal expression occurred only at T4, while Lus 49 showed induction only at T12 (Figure 4g).

3.7 | Expression Study of P Deficiency Tolerance Genes

Expression of linseed genes under Pi deficiency was analyzed in the shoot tissues of three genotypes. *LusACA3687* was differentially expressed in Lus 29, showing slight downregulation under low Pi (Figure 5b). *LusACA3300* showed no change in Lus 29 and was undetected in Lus 37 and Lus 4 (Figure 5a). *LusACA3736* was expressed only under optimum Pi in Lus 37, with no variation in Lus 4 and Lus 29 (Figure 5c). *LusACA3522* was expressed only under low Pi in tolerant Lus 37, while Lus 29 showed moderate downregulation, and Lus 4 showed no expression (Figure 5d).

LusPT6826 expression appeared only under optimum Pi in Lus 37, with none detected in Lus 29 or Lus 4 (Figure 5e). *LusPT6548* was eightfold downregulated under low Pi in Lus 29, and undetected in Lus 37 and Lus 4 (Figure 5f). *LusPT6635* showed 18-fold downregulation in Lus 29, while Lus 37 showed no change and Lus4 showed no expression (Figure 5h).

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TABLE 1 | Details of the orthologs identified.

Sl No.	Genes	Locus ID	Location	e- value	Traits	PFAM	PFAM type of the selected gene
1	*MATE1*	Lus10039609	Scaffold15	0	Al^{3+} toxicity	PF01554	PF01554
		Lus10029517	Scaffold55	0		PF01554, PF13456	
		Lus10016412	Scaffold179	0		PF01554	
		Lus10026303	Scaffold898	0		PF01554	
		Lus10042365	Scaffold123	0		PF01554	
2	*ALMT1*	Lus10021272	Scaffold1123	0		PF11744	PF11744
		Lus10013577	Scaffold62	0		PF11744	
3	*MGT1*	Lus10014353	Scaffold275	0		PF01544	PF1544
		Lus10026063	Scaffold319	0		PF01544	
		Lus10021887	Scaffold164	60e-84		PF01544	
		Lus10015459	Scaffold860	98e-81		PF01544	
4	*PT6*	Lus10016635	Scaffold915	0	Pi deficiency	PF07690	PF00083
		Lus10022548	Scaffold465	0		PF00083	
		Lus10011826	Scaffold610	0		PF07690	
		Lus10014754	Scaffold584	0		PF07690	
		Lus10021191	Scaffold11	0		PF07690	
		Lus10024884	Scaffold473	96e-164		PF00083	
5	*PAP15*	Lus10011847	Scaffold754	0		PF16656, PF00149, PF14008	PF16656, PF00149, PF14008
		Lus10022786	Scaffold8	0		PF16656, PF00149, PF14008	
		Lus10016667	Scaffold915	0		PF16656, PF00149, PF14008	
		Lus10016665	Scaffold915	0		PF16656, PF00149, PF14008	
		Lus10028533	Scaffold413	0		PF16656, PF00149, PF14008	
6	*SPL4*	Lus10016275	Scaffold947	67e-139		PF03110	PF03110
		Lus10012020	Scaffold931	52e-162		PF03110	
7	*WD40 − 38*	Lus10043302	Scaffold25	0		PF00400	PF00400
		Lus10019450	Scaffold906	0		PF00400	
8	*ACA3*	Lus10011522	Scaffold260	0		PF00689, PF00122, PF13246, PF00690, PF12515	PF13246, PF00689, PF00690, PF00122, PF00702
		Lus10019300	Scaffold377	0		PF00689, PF00122, PF13246, PF00690, PF12515	
		Lus10007736	Scaffold993	0		PF00689, PF00122, PF13246, PF00690, PF12515	
		Lus10025199	Scaffold305	0		PF00689, PF00122, PF13246, PF00690	
		Lus10018687	Scaffold461	0		PF00702, PF00689, PF00122, PF13246, PF00690, PF12515	

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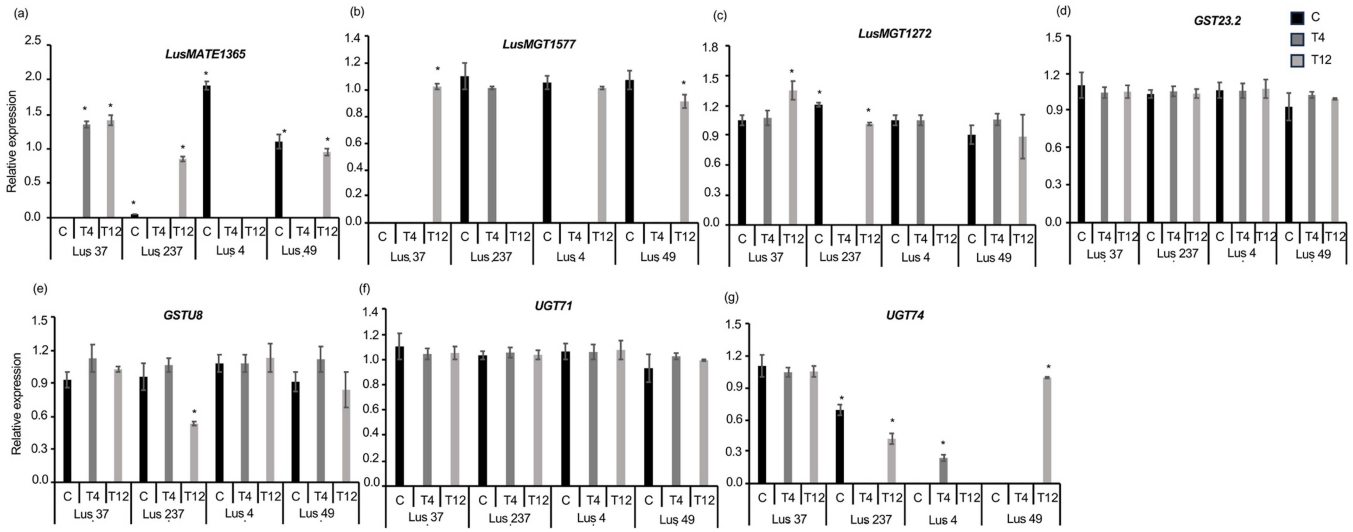


FIGURE 4 | Time-course expression of aluminum-responsive genes in linseed root tissues. Expression level of (a) *LusMATE1365*; (b) *LusMGT1577*; (c) *LusMGT1272*; (d) *LusGSTU8*; (e) *LusGST23.2*; (f) *LusUGT74* and (g) *LusUGT71* genes in the root tissues of four genotypes (Lus 4, Lus 37, Lus 49 and Lus 237) in response to different time interval exposures (control (C) – 0 h, T4 – 4 h, T12 – 12 h) to 500 μM Al^{3+}. *EF1A* was used as a reference gene for normalization, and the relative values were plotted. The data represent the mean ± SD of three biological and three technical replicates. The Y-axis represents the relative expression, and the X-axis represents the genotypes. Significant expression differences at $p < 0.05$ between the C and T for a particular genotype were assessed using one-way ANOVA and are represented with asterisks (*) in the bar diagram.

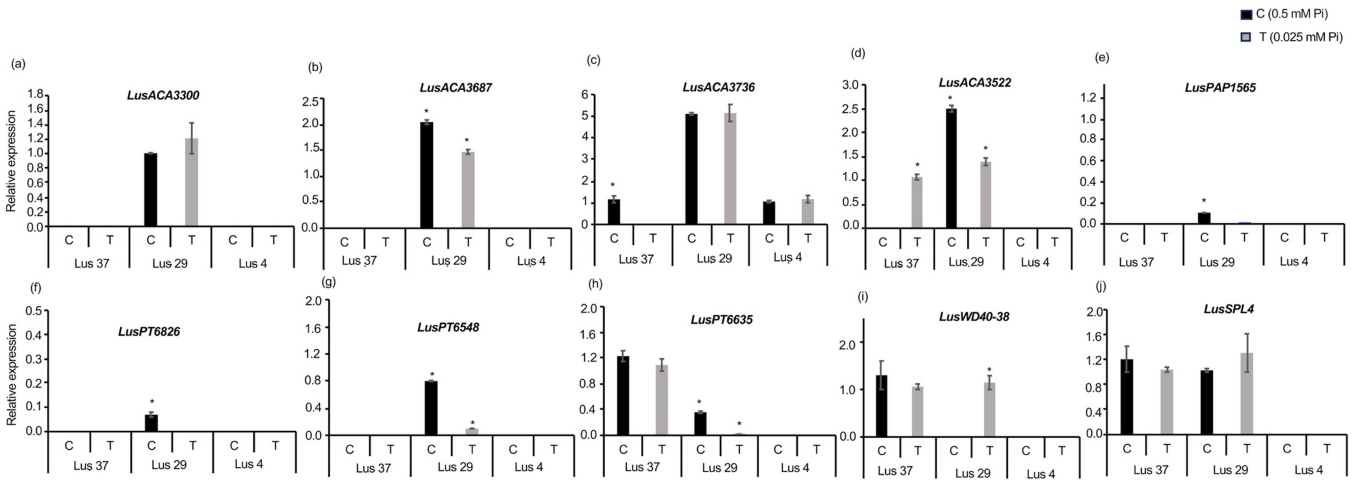


FIGURE 5 | Expression of phosphorus-responsive genes in linseed shoots under Pi deficiency. Expression level of (a) *LusACA3300*; (b) *LusACA3687*; (c) *LusACA3736*; (d) *LusACA3522*; (e) *LusPAP1565*; (f) *LusPT6826*; (g) *LusPT6548*; (h) *LusPT6635*; (i) *LusWD40-38* and (j) *LusSPL4* genes in the shoot tissues, harvested after 14 days of treatment across the three genotypes (Lus 4, Lus 29, and Lus 37) in response to Pi concentrations of control (0.5 mM) and treatment (0.025 mM). *EF1A* was used for normalization, and the relative values were plotted. The data represent mean ± SD of three biological and three technical replicates. The Y-axis represents normalized relative expression, and the X-axis represents the genotypes. Significant expression differences at $p < 0.05$ between the C and T for a particular genotype were assessed using one-way ANOVA and are represented with asterisks (*) in the bar diagram.

LusWD40-38 was expressed only under low Pi in Lus 29, with similar levels in both treatments for Lus 37 and no expression in Lus 4 (Figure 5i). *LusSPL4* showed no differential expression in Lus 29 and Lus 37, and none in Lus 4 (Figure 5j). Basal expression of *LusPAP1565* was detected only in Lus 29 (Figure 5e). In root tissues, *LusACA3687* was expressed only under optimum Pi in Lus 29 and Lus 4, with no expression in Lus 37 (Figure 6b). *LusACA330* and *LusACA3199* were detected only under optimum Pi in Lus 29 (Figure 6a, d). *LusACA3736* was slightly downregulated under low Pi in Lus 4, and undetected in Lus 29 and Lus 37 (Figure 6c). *LusPT6826* showed basal expression under low Pi in tolerant Lus 37, while susceptible lines expressed it only under optimum Pi (Figure 6e). *LusPT6548* appeared only under optimum Pi in Lus 29 (Figure 6f). *LusPT6635* was highly induced under 14-day low Pi in tolerant Lus 37, while Lus 29 showed 76.2-fold downregulation and Lus 4 showed no change (Figure 6g). Both *LusWD40*-38 and *LusSPL4* were expressed only under optimum Pi in susceptible genotypes, with no expression observed in Lus 37 (Figure 6h,i). While several genes showed differential expression under Al^{3+}

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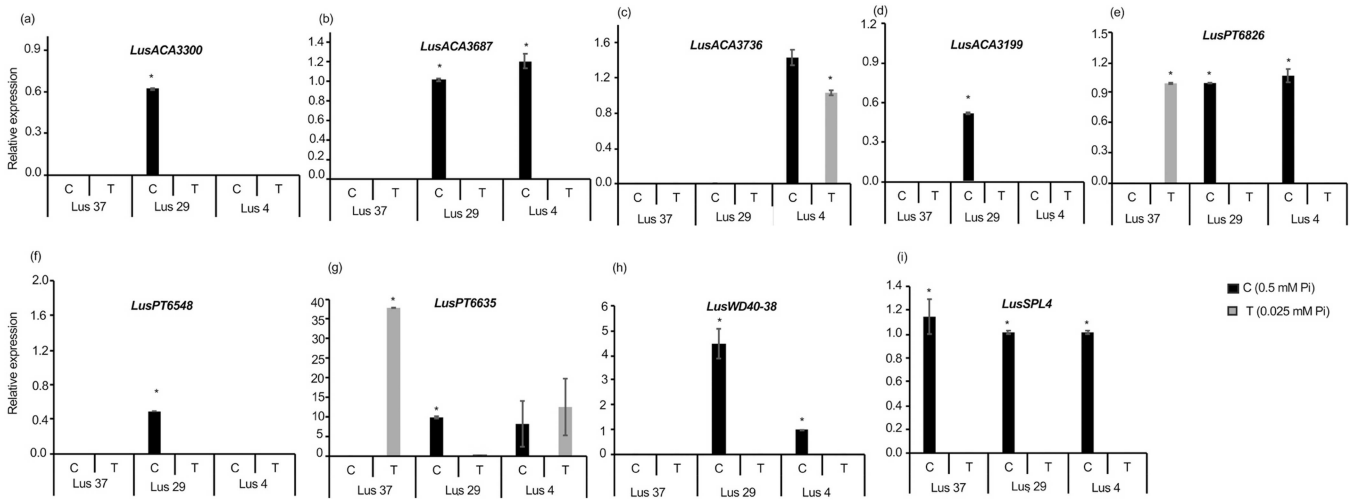


FIGURE 6 | Expression of phosphorus-responsive genes in linseed roots under Pi deficiency. Expression levels of (a) *LusACA3300*; (b) *LusACA3687*; (c) *LusACA3736*; (d) *LusACA3522*; (e) *LusPAP1565*; (f) *LusPT6826*; (g) *LusPT6548*; (h) *LusPT6635*; (i) *LusWD40-38* and (j) *LusSPL4* genes in the root tissues harvested after 14 days of treatment across the three genotypes (Lus 4, Lus 29 and Lus 37) in response to Pi concentrations of control (0.5 mM) and treatment (0.025 mM). *EF1A* was used for data normalization, and the relative values were plotted. The data represent mean ± SD of three biological and three technical replicates. The Y-axis represents the normalized relative expression, and the X-axis shows the genotypes. Significant differences at $p < 0.05$ between control and treatment for a given genotype were assessed using one-way ANOVA and are represented with asterisks (*) in the bar diagram.

toxicity and Pi deficiency, some exhibited weak, irregular, or marginal fold changes across genotypes and tissues (e.g., *LusACA3522* and *LusWD40-38*), particularly in susceptible genotypes. Such variable expression suggests a context-dependent or secondary role in stress responses rather than a primary causal function. Accordingly, downstream interpretation was limited to genes showing consistent and biologically meaningful regulation across tolerant genotypes, including *LusMATE1365*, *LusUGT74*, and *LusPT6635*, which were prioritized as key candidate genes for Al^{3+} toxicity and Pi deficiency tolerance in linseed.

3.8 | Correlation Between Transcriptome Data and qRT-PCR

A correlation analysis was conducted to compare the available transcriptome data from fiber-type linseed with data generated from oilseed-type linseed, aiming to identify shared genes in both linseed types. Expression of *MATE1365* in Hermes, a resistant fiber-type genotype, showed a strong correlation with Lus 37, a tolerant oilseed-type genotype, at a significance level of 1%. The expression of *MGT1577* in TM1919, a resistant fiber-type genotype, indicated a correlation at a 1% significance level with Lus 37. In the sensitive fiber-type genotype Orshan, the expression of *MGT1577* was correlated at a significance level of 0.1% with Lus 4 and Lus 49, both of which are susceptible oilseed genotypes. Additionally, the susceptible fiber-type genotype Lira exhibited significant correlations at the 0.1% and 1% levels with Lus 4 and Lus 49, respectively. The expression of *MGT1272* in Hermes indicated a correlation at 1% level of significance with Lus 37 and Lus 237, both of which are tolerant oilseed-type genotypes. While expression of *MGT1272* in TM1919 indicated a 0.1% level of significance with Lus 237. No correlation for low Pi-responsive genes between the fiber-type and oilseed-type genotypes was found.

4 | Discussion

4.1 | Rationale for Identifying Al^{3+} Toxicity and Pi Deficiency Tolerance Genes and Genotypes

Pi deficiency and Al toxicity in acid soils are a problem for productivity of linseed (Bezil et al. 2025). Therefore, identification of varieties that are tolerant to Pi deficiency and Al^{3+} toxicity is needed for increasing the crop yield. It is important to study genotypic variations in terms of morphological, physiological, and molecular responses to these stresses. In the NEHR of India, soil acidity frequently leads to Al^{3+} toxicity and Pi deficiency, thereby limiting crop establishment and productivity (Zheng 2010; Tyagi and Rai 2012; Liu et al. 2022). Targeted identification of linseed genotypes adapted to acidic soils is critical for improving rabi-season productivity in rice–fallow systems of NEHR.

Linseed-specific tolerance mechanisms were investigated by identifying contrasting genotypes and analyzing their phenotypic and molecular responses. The acidic soil in the rainfed rabi season primarily suffers from Al^{3+} toxicity and low Pi conditions. Genotypes adapted to acidic soils must combine tolerance to both Al^{3+} toxicity and low Pi availability, as these stresses co-occur under field conditions. As both these traits are polygenic, genes conserved across species but differentially regulated within linseed genotypes offer promising targets for improving field performance. In this study, the contrasting genotypes identified by soil screening were re-assessed under control conditions using hydroponics. This step was followed by the qPCR experiment to understand the expression pattern in low Pi and Al^{3+} toxicity conditions. Roots were analyzed for Al^{3+} toxicity, and roots plus shoots for low Pi to capture tissue-specific responses in linseed.

Hydroponic experiment provides a rapid and reliable method for the identification of the contrasting genotypes with easy observation and minimal influence of environmental factors.

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The most used parameters for assessing Pi deficiency and Al^{3+} toxicity tolerance are root biomass and root length (Liu et al. 2004; Zhou et al. 2013). Under low Pi conditions, tolerant genotypes showed enhanced root surface area and maintained shoot biomass, suggesting improved phosphorus-use efficiency. Tolerant genotypes under Pi deficiency showed significantly increased root biomass and R/S ratio (Zheng et al. 2021). Such mechanisms (Tyagi and Rai 2017; Bernardino et al. 2019; Kothari et al. 2023) are likely to contribute to tolerance in linseed, although functional validation is required. In this study, six oil-seed type linseed genotypes were evaluated under hydroponic conditions, and genotypes Lus 37 and Lus 237 showed less reduction in root length and root biomass as compared to Lus 4, Lus 29, and Lus 49 and were considered tolerant. The results are similar to those reported in maize (Kong et al. 2020). The root growth inhibition, root biomass, and visualization of haematoxylin stain in the treated root samples were taken as indirect traits for identification of tolerance to Al^{3+} toxicity in rice (Awasthi et al. 2017; Tyagi et al. 2020). Consistent with these studies, linseed genotypes Lus 37 and Lus 237 exhibited reduced root growth inhibition and weaker haematoxylin staining under Al^{3+} toxicity. Based on the root biomass and haematoxylin staining, genotypes Lus 4 and Lus 49 were considered as susceptible and Lus 37 and Lus 237 as tolerant under Al^{3+} toxicity conditions. These phenotypic distinctions provided a robust framework for subsequent expression analysis in linseed (Supporting Information Table S2). Genes previously implicated in Al^{3+} toxicity and Pi deficiency tolerance in cereals (Tyagi et al. 2021) and soybean (Wang et al. 2009) provide a reference framework for selecting linseed orthologs for expression analysis.

4.2 | Expression Pattern Analysis for Linseed Orthologs to Identify Candidate Genes With a Role in Imparting Al^{3+} Toxicity and Pi Deficiency Tolerance

Differential expression pattern of genes reported for imparting tolerance to Al^{3+} toxicity and Pi deficiency has been reported in several plant species, including fiber-type linseed (Liu et al. 2014; Dmitriev et al. 2016a; Krasnov et al. 2019; Sasaki et al. 2006; Maron et al. 2010; Duan et al. 2022). In oil-seed type linseed, upregulation of *MATE1* and *UGT74* in the tolerant genotype Lus 37 under Al^{3+} toxicity suggests conservation of organic anion–mediated detoxification mechanisms. A significantly higher expression of *MATE* was observed in the root tip tissues of Al-tolerant soybean and chickpea under high Al^{3+} (Liu et al. 2016; Zhang et al. 2020).

The upregulation of *GST* genes and the defense against the Al^{3+} toxicity via *GST* antioxidant activity is one of the mechanisms for the fiber-type flax tolerant to Al^{3+} stress (Dmitriev et al. 2016b). In the present study, *GST*- and *UGT*-associated responses were observed primarily in tolerant oil-seed linseed genotypes, although fold changes were moderate. Upregulation of *GST* and *UGT* only in tolerant genotypes across species, including fiber-type linseed genotypes, was reported. Besides, the expression of genes imparting tolerance was found to be higher under Al^{3+} treatment in the resistant genotypes than in the sensitive ones (Kochian et al. 2015). A similar expression pattern was observed in the tolerant genotype, Lus 37, for genes like *MATE1* and *UGT74* but the genotype, Lus 237, showed a different expression pattern. The mechanism of tolerance in response to high Al^{3+} in the tolerant genotype, Lus 37, appears to be similar to that reported in other species, including fiber-type linseed. Additionally, *MGT1272* and *MGT1577* expression in oil-type linseed was also similar to *UGT74*. So, the mechanism for Al^{3+} toxicity tolerance reported in other crops and fiber-type linseed is similar to the oil-seed type linseed, at least for these genes in the genotype Lus 37.

PAP15 is strongly induced by Pi deficiency in roots of Brassica and tobacco, and in both roots and shoots of rice (Zamani et al. 2012; Du et al. 2025). In linseed, *PAP15* expression was limited and genotype-specific, suggesting divergence from model crop responses.

OsPT6 is predominantly expressed in the roots and leaves in response to Pi starvation and is a high-affinity phosphate transporter needed for the enhanced phosphorus efficiency (Zhang et al. 2014). Upregulation of linseed orthologs *PT6826* and *PT6635* in roots of Lus 37 supports their role in Pi acquisition under deficiency. Overexpression of phosphate transporters in rice results in an increase in the uptake of inorganic phosphate from the soil and its translocation within the plants. Upregulation of orthologs, *PT6826* and *PT6635*, in the roots of the tolerant genotype, Lus 37, with downregulation in shoots of the susceptible genotype, Lus 29, in linseed, is similar to reports in rice.

In rice, *WD40-38*, *SPL4*, and *ACA3* are upregulated under low Pi, and overexpression of phosphate transporters enhances Pi uptake and translocation. In linseed, orthologs *PT6826* and *PT6635* were upregulated in roots of the tolerant Lus 37 but downregulated in shoots of susceptible Lus 29, while *WD40-38*, *SPL4*, and *ACA* orthologs showed genotype- and tissue-specific downregulation in susceptible genotypes, indicating crop-specific regulatory responses to Pi deficiency.

5 | Conclusion

Oilseed-type linseed genotypes were evaluated for tolerance to Al^{3+} toxicity and Pi deficiency, major constraints of acidic soils. Lus 37 and Lus 237 consistently exhibited reduced root growth inhibition under Al^{3+} toxicity, whereas Lus 4 and Lus 49 were susceptible. Lus 37 also performed better under low Pi, indicating genotype-specific adaptive capacity. An orthology-based analysis identified candidate genes from eight gene families previously implicated in Al^{3+} and Pi deficiency tolerance. The stress-responsive regulation of *LusMATE1365*, *LusUGT74*, and *LusPT6635* in tolerant genotypes suggests their potential role in linseed adaptation to acidic soils. Inconsistent expression of some transcriptional regulators highlights the importance of crop-specific validation. This study provides linseed-specific phenotypic and molecular evidence for differential tolerance and identifies promising genotypes and candidate genes for functional validation and breeding aimed at improving productivity in acidic soils of the NEHR. The candidate genes identified in this study (e.g., *LusMATE1365*, *LusUGT74*, *LusPT6635*) provide a foundational framework for improving linseed tolerance to Al^{3+} toxicity and Pi deficiency. Future work should focus on the functional validation of these genes using approaches such as gene editing targeting coding or regulatory

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regions, to confirm their roles in stress tolerance. Additionally, evaluating these genes across diverse germplasm and in field conditions will be critical to assess their effectiveness for breeding applications. Integration of these validated genes into molecular breeding pipelines could accelerate the development of linseed varieties adapted to acidic soils.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. The data that support the findings of this study are included as supplementary data.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Figure S1: Diagrammatic representation along with screenshot showing methodology for finding putative linseed orthologs, expression profiling and primer design using Phytozome, FlBexDB and NCBI primer BLAST tool. **Table S1:** List of contrasting genotypes screened and identified for expression studies of Al toxicity and Pi deficiency responsive genes. **Table S2:** Details of the genes selected for the study. **Table S3:** Details of orthologs identified. **Table S4:** Primer list.

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