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Expression Analysis of *Puccinellia tenuifolra* Gene *PutSTE24* and Response to Aluminium Stresses

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Abstract Alkaline grass (*Puccinellia tenuiflora*) can serve as a model of salt tolerance in monocotyledon crops. However, little is known about its mechanism of aluminum stress. In this work, we screened *PutSTE24* gene under aluminum stress to predict its subcellular localization of proteins by using bioinformatics analysis software, and the result showed that this gene was in the endoplasmic reticulum, which was the same with expressing *PutSTE24* gene in yeast INVsI. Under aluminum and saline-alkali stress conditions, the relative mRNA expression levels of the *PutSTE24* of alkaline grass were measured by real-time quantitative PCR, and the results demonstrated that its expression was induced up regulation. We also constructed a plant expression vector of *PutSTE24* gene, and then transferred into Arabidopsis. For further determine the correlation between *PutSTE24* gene and aluminum stress, we choose transgenic seeds for resistance experiment, which provided a wealth of theoretical and experimental basis for further research on interaction of related genes and regulatory mechanism of aluminum stress.

Keywords *Puccinellia tenuifolra*; *PutSTE24* gene; Aluminum stresses; Transgenic Arabidopsis

Introduction

Plants may be subjected to kinds of abiotic stresses in the process of growth. Aluminum toxicity and low pH is a major environmental factor limiting plant growth and production on acid soils and 50% of the world's arable land is under the threat of soil acidification (Shinozaki et al., 2003). Aluminum toxicity may be triggered in both acid and alkaline conditions due to the complex chemistry of Al. The trivalent Al species (Al^{3+}) dominates in acid conditions (pH < 5.5), whereas $Al(OH)^{4-}$ in alkaline conditions and an extremely toxic polynuclear Al species, Al_{13} can also form when Al solutions are partially neutralized with a strong base. All those Al species can inhibit root growth and then limit the growth and production, so it's of great value to clarify the molecular mechanism of aluminum toxicity in the saline-alkalized land (Foy, 1988). Aluminum of trace amount can promote growth of plants to some degree (Peng et al., 2003), but it turns to be toxic when the concentration increases to the range of micromole(Doncheva et al., 2005). Studies on maize have indicated that the high concentration of aluminum Al^{3+} can inhibit cell divisions in short time (Matsumoto 2000). The most obvious symptoms of the plants being cultivated with the micromolar concentration of Al^{3+} is the inhibition of the elongation of root (Zhang and Rengel, 1999), and the root become short and thick (Horst 1995). The root apex accumulates more Al and suffers greater physical damage than the other parts of the root (Foy et al., 1967). The aluminum toxicity can not only hinder minerals absorption and restrain cellular metabolism (Kollmeier et al., 2000), but also break the balance of hormone and exert influence on activities of enzymes, and inhibit mitosis and the synthesis (Matsumoto, 2000). As far as the cells and tissues are

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concerned, aluminum toxicity can destroy the structure and function of the membrane(Zhang et al., 1997) and the stability of the cell wall (Matsumoto, 2000) and cytoskeleton (Sasaki et al., 1996), and it will be an inhibition of cell-elongation (Willats et al., 2001). From the level of organs ,it performed to inhibit the growth of plant root, stem, leaf (Ryan et al., 1993; Samuels et al., 1997; Sivaguru and Horst, 1998), and prompt tissue necrosis(Shen and Yan, 2001). In recent years, the researchers cloned a series of genes associated with aluminum toxicity. At the molecular level there has a certain explanation how plant response the aluminum toxicity (Yang et al., 2004), however the main stress mechanism of aluminum toxicity in plant is still unknown. *Puccinellia tenuifolra*, *puccinellia* of gramineous, also known as the star grass, it is a perennial herbs that mainly distributed in saline-alkali land in the northeast China plain. Because of its strong adaptability, *puccinellia* can grow in the extremely severe salinization of soil, and even in pH up to 11 of the soil it can also be normal growth. *Puccinellia* is important to restore the saline soil vegetation (Wang and Guo, 2001), but at present the research on mechanism of aluminum toxicity in *puccinellia* was not reported. This study screened *PutSTE24* gene of *puccinellia* with aluminum resistance. Using biology software to predict the subcellular localization of this gene, using yeast *InvsI* to express *PutSTE24* gene to clarify the positioning results. By real-time fluorescent quantitative PCR technique to detect *PutSTE24* mRNA expression features. Construction of plant expression vector to transform *Arabidopsis thaliana*, take advantage of resistance experiment in seed germination to confirm the relationship between *Puccinellia* and aluminum toxicity. This study laid the foundation for further researching the interactions between *PutSTE24* and the related gene of *Puccinellia* and regulatory mechanism.

1 Results and Discussion

1.1 Homologous comparison analysis of *PutSTE24* gene

cDNAs library of *Puccinellia* has been done by our lab, we obtained the *PutSTE24* gene KJ598490 length is about 1 800 bp under the aluminum resistance screening, coding frame is 1 275 bp, coding protein size is 424 amino acid, predict the molecular weight of 48.3 kD. The isoelectric point is 6.84. According to the NCBI, using the BLAST search tool, we identified an expressed sequence of *PutSTE24*. We can find higher homology 12 species such as *Brachypodium distachyon*. Using MEGA software to analyze the evolutionary tree (Figure 1), the evolutionary tree is in order to resolve the relationship between genes and other species. Results show that the *Puccinellia PutSTE24* shares 90% identity with *Brachypodium distachyon* and barley, 80% identity with rice, sorghum, and maize, 70% identity with this *CAAX* protease of *STE24* in *Arabidopsis thaliana* and canola.

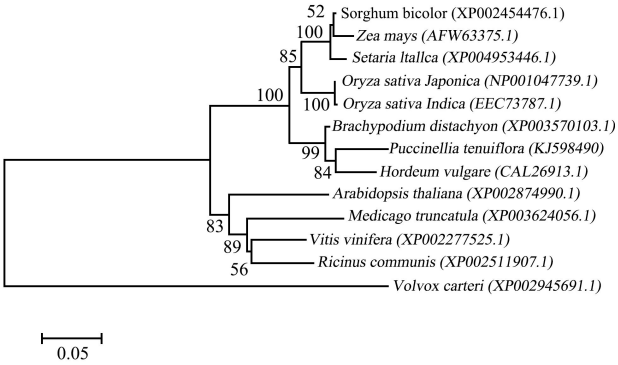

Figure 1 Phylogenetic tree analysis of *PutSTE24* gene

Note: Bootstrap values are calculated 1 000 times; values below 50 % are not included

1.2 Subcellular localization of converting *PutSTE24* gene of yeast cells

Through bioinformatics software to predict the positioning of *Puccinellia PutSTE24* genes encode proteins within the cells. The results show that the *PutSTE24* genes encode proteins is utmost in the endoplasmic reticulum (Figure 2A). At the same time, this study further used the Polyethylene glycol (PEG)/lithium acetate (PEG/LiAc) to transform *thepYES2*: *GFP*, *pYES2*: *PutSTE24*: *GFP* into wine yeast in *INVScI*. After galactose induced by laser confocal microscopy to detect the fluorescence. We can observe the control *pYES2*::*GFP* abundantly expressed in the cytoplasm in yeast (Figure 2, *pYES2*), and *PutSTE24*:: *GFP* fusion protein showed a *GFP* fluorescence can be observed (Figure 2, *pYES2-PutSTE24*), and we can also see the cell

structure that present similar circular or oval, it is a typical structure of the endoplasmic reticulum, at the same time we combined with the predictions of a biological software, presumably the genes that are located inendo plasmic reticulum. According to the *AtSTE24* of Arabidopsis that are located in the endoplasmic reticulum and it is the homologous gene with *PutSTE24* genes of *Puccinellia*. And in other yeast and animal cells, the CAAX protein located in endoplasmic reticulum (Dai et al., 1998; Hildebrandt et al., 2013; Manolaridis et al., 2013), so you can preliminary confirmed that the *PutSTE24* gene located in the endoplasmic reticulum.

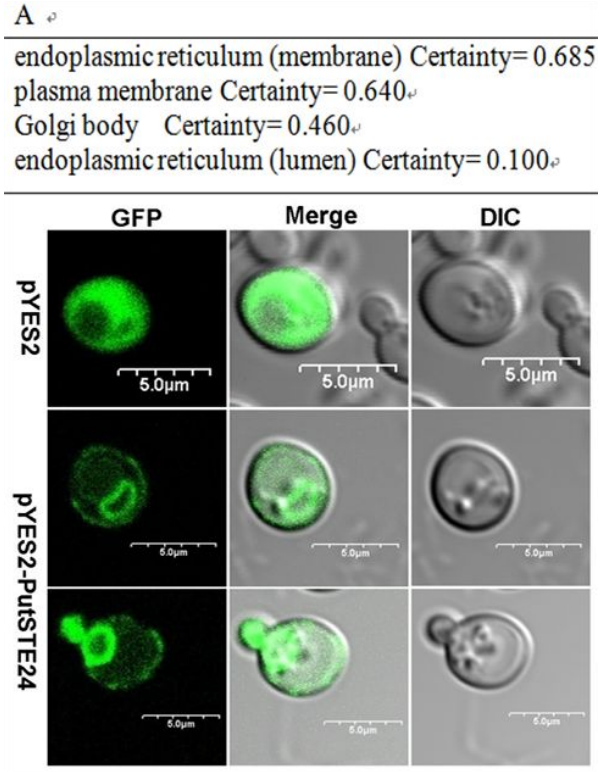

Figure 2 The subcellular predication of the deduced *PutSTE24* and *PutSTE24* located in yeast cells

Note: A: The subcellular predication of the deduced *PutSTE24*, (*pYES2*)Yeast cells which expressed GFP as a control, fluorescent, merged vision and bright; (*pYES2-PutSTE24*) Yeast cells which expressed *PutSTE24*::GFP, fluorescent, merged vision and bright

1.3 *PutSTE24* mRNA expression in stress analysis

To investigate correlation of *PutSTE24* and abiotic stress response, the real-time fluorescent quantitative PCR was used to analyze gene expression characteristics under aluminum poisoning and salinity stress. The result showed that the expression of *PutSTE24* significantly was induced by $AlCl_3$, NaCl and $NaHCO_3$ stress. Firstly, the expression of *PutSTE24* in different tissues and organs (root, stem, leaf, flower and sheath) was analyzed using qPCR. The results displayed the expression level of *PutSTE24* was the highest in root, then the second is in leaf and sheath, and the expression level was least in stems and flowers. The expression of root was about 3 times of stems and flowers, and the expression of leaves and sheath was about 2 times of stems and flowers (Figure 3). The homologous gene of *Arabidopsis AtSTE24* in various tissues expression characteristic analysis showed the stem and leaf expression of *AtSTE24* was similar with *PutSTE24*, and leaf expression was also higher than stem (Bracha et al., 2002).

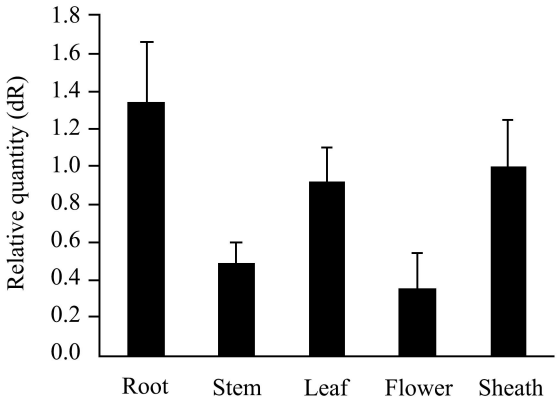

Figure 3 Real-time quantitative expression analysis of *PutSTE24* tissue specificity in *puccinellia* (root, stem, leaf, flower and sheath)

In 100 μM $AlCl_3$ treatment, the expression of *PutSTE24* in root increased in the processing time, and the expression reached the highest in 48 h. It was about 8 times with the control (Figure 4A). The expression had a large amount of growth in the leaf from 6 h, which was about 4-fold higher than the contrast. The expression slightly decreased in 12 h, but still higher than control. Thus the expression was the least within 24 h, which was not different with the control. But the expression level increased in 48 h (Figure 4B). The expression of *PutSTE24* reached its

highest value at 6 h in the 100 mM NaCl treatment and was 5 fold higher than that of the control. Subsequently, the expression declined gradually at 12 h, 24 h, and 48 h, and remained similar with the control (Figure 4C). In NaCl treatment, the expression increased in 6 h, then decreased in 12 h and the expression rose in 24 h, finally was slowly decreased in 48 h, but the expression is about 1.5 times of the control (Figure 4D). The results of analyses showed that the expression level of *PutSTE24* changed after 80 mM $NaHCO_3$ treatment and that the degree of the change was in direct proportion to the treated time (Figure 4E). Compared with the control (0 h), the expression rose at 6 h and 12 h treatments. However, the expression clearly increased at 24h treatment. The highest expression occurred at 48 h, which was approximately 7-fold higher than that of the control. The expression was detected at different times, and the results are shown in (Figure 4F). The expression level did not change compared with that of the control group. The highest value appeared at 6 h, and the change was significant. The results showed that *PutSTE24* can be induced by $AlCl_3$, NaCl and $NaHCO_3$ stress. Especially, the gene expression of root greatly increases with the increase of time under $AlCl_3$ and $NaHCO_3$ stress, which was about 7.5 times greater than that of the control. In $AlCl_3$, NaCl and $NaHCO_3$ stress, the gene expression of leaf began to increase in 6 h, but had a slight decline in 12 h, while the expression rose again in 24 h and 48 h. It was suggested that *PutSTE24* is the related gene with $AlCl_3$, NaCl and $NaHCO_3$ treatments, may participate in adversity stress response and improve the resistance.

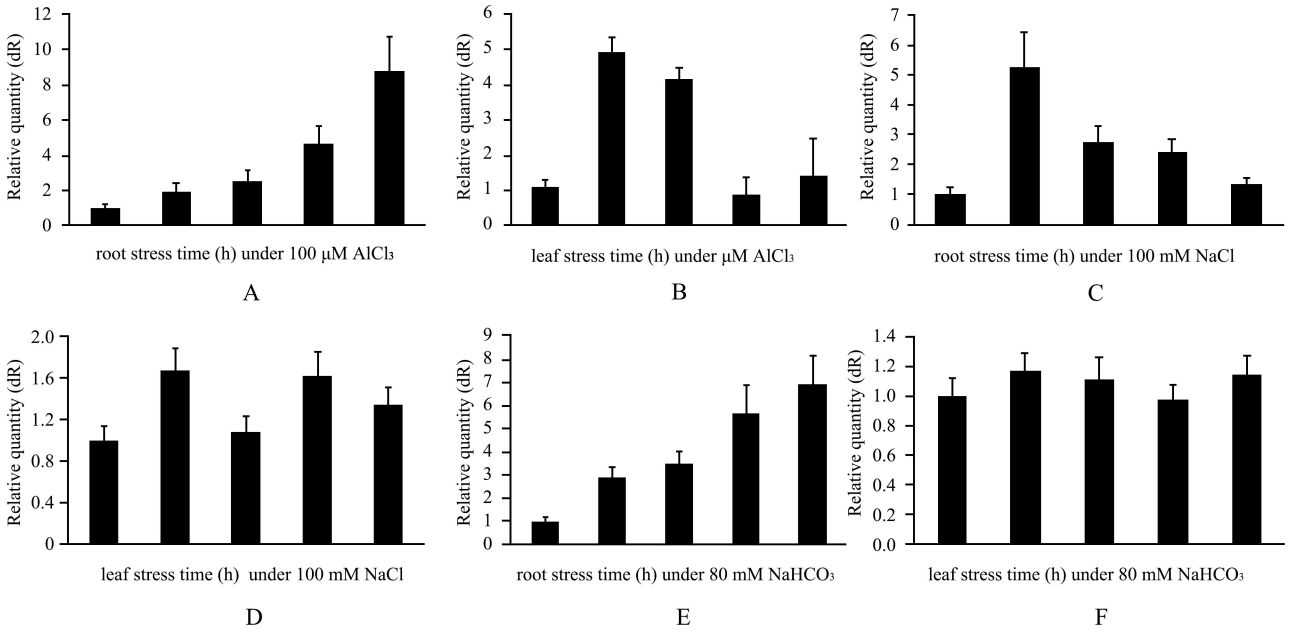

Figure 4 Real-time quantitative expression analysis of PutSTE24 gene under stresses of aluminum ($AlCl_3$), salt (NaCl), alkali ($NaHCO_3$)

Note: A, B: Expression change of gene treated with 100 μmol/L $AlCl_3$; C, D: Expression change of gene treated with 100 mmol/L NaCl; E, F: Expression change of gene treated with 80 mmol/L NaHCO3

1.4 Overexpression of *PutSTE24* gene tolerance analysis in Arabidopsis

PutSTE24 overexpressing transgenic lines (3#, 4#, 5#, 6#, 8#) expressing the full-length *PutSTE24* cDNA from the cauliflower mosaic virus (CaMV) 35S promoter were generated, tested, and confirmed to accumulate the PutSTE24 by northern-blot analysis. The results showed that transformation of T2 generation of Arabidopsis (T2-4#, 5#, 6#) strain can be detected (Figure 5). The growth of wild-type (WT)

was similar with the overexpressors. But the phenotype of WT and overexpressors were suppressed after $AlCl_3$ treatments. The root became short and the growth of leaf is restrained. The overexpressing transgenic lines growth of root and leaf was significantly better than that of WT under 200 μm, 300 μm and 400 μm $AlCl_3$ treatment (Figure 5). Under 400 μm $AlCl_3$ (Figure 5D), WT cannot grow, while overexpressors still can grow. Meanwhile, fresh weight of overexpressors distinctly was higher than WT in 300 μm and 400 μm $AlCl_3$. The results suggested that *PutSTE24* overexpressing transgenic had better tolerance of $AlCl_3$ than WT. So *PutSTE24* may be induced to alleviate the Al toxicity.

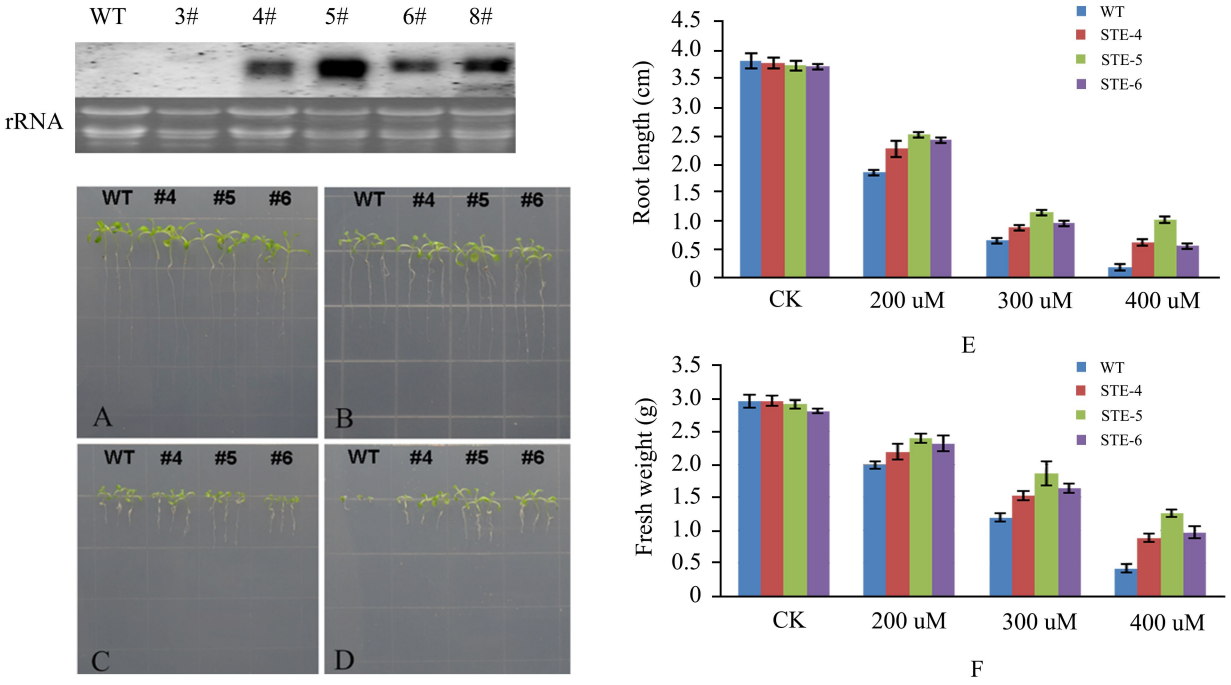

Figure 5 Resistant analysis of transgenic Arabidopsis over-expressing *PutSTE24* in Al-stress

Note: Northern blot analysis was used to test expression of *PutSTE24* and transgenic Arabidopsis in control and Al-stress. A: WT and transgenic seeds germinated in 1/2MS solid medium agar plates for 14-day-old; B: 200μM $AlCl_3$ toxicity; C: 300 μM $AlCl_3$ toxicity; D: 400 μM $AlCl_3$ toxicity; E: Detection of root growth; F: Detection of fresh weight

2 Material and Methods

2.1 Material

The cDNAs library was constructed and preserved in the Alkali Soil Natural Environmental Science Center (ASNESC), Northeast Forestry University, and Harbin, China. Alkaline grass seeds were cultured with hydroponics. The leaf and root were collected and cryopreservation in -80℃ for RNA extraction.

2.2 Sequence analysis

The analysis of complete nucleotide sequence of *PutSTE24* was performed using Blast X in the nonredundant aminoacid sequence database of the NCBI website (http://www.ncbi.nlm.nih.gov). The open reading frame (ORF) and the deduced amino acid sequence were analyzed using DNA star v7.1 software (http://dnastar.com/t-products-lasergene.aspx), to examine the alignments of multiple sequences (Larkin et al., 2007). The maximum likelihood (ML) phylogenetic tree was constructed in the PHYML website (http://atgc.lirmm.fr/phyml/).

2.3 Construction of expression vector and transformation

To understand the subcellular localization of *PutSTE24*, we predicted its encoded proteins by using bioinformatics analysis software (Prediction of Protein Localization Sites version 6.4) this gene was

amplified from constructed cDNA library. The correctly modified gene was inserted into yeast expression vector *pYES2* to construct *pYES2-PutSTE24-GFP*, and then the expression vector was transformed into saccharomyces cerevisiae strains *INVScI*. After induced by galactose, yeast was detected fluorescence expression by laser scanning confocal microscope for further clarify the subcellular localization of *PutSTE24*.

2.4 Expression analysis of *PutSTE24* under aluminum stress condition

Alkaline grass seeds were pre-cultured with hydroponics for two weeks, then they were cultured in 100 mmol/L NaCl, 80 mmol/L $NaHCO_3$ and 100 μM $AlCl_3$. Leaf and root were collected at regular intervals (6 h, 12 h, 24 h, 48 h), and then extracted the total RNA based on the practice of the literature. CDNA was synthesized by reverse transcription according to the manufacturer's instructions of First-Strand cDNA Synthesis Kit (TakaRa, China). RT-PCR was performed using MxPro-Mx3000P to detect the mRNA expression levels of *PutSTE24* gene under different stress conditions and different tissues. A pair of gene *PutSTE24* specific primers, F: 5'-ACACCCTTGCGTTCTTAGCAGG-3', R: 5'-AGCCACAATTGGCGGTGCGA G-3' was used to amplify a DNA fragment of 1 275 bp. Another pair of specific primers for the actin of *Puccinellia tenuiflora*, 5'-TTGAACAAGAAATGGCAACTGCTG-3' (actin-F) and 5'-CAAGGAAAGATGGTTGGAAAAGTG-3' (actin-R), was used to amplify a DNA fragment of 1 275 bp as the internal control. The PCR reaction was performed in a 20 μL centrifuge tube containing 2×BrilliantⅢ SYBR Green QPCR Master Mix (Agilent), 1 μL of cDNA, 1 μL each of the forward and reverse primers (10 μM). The reaction procedure was performed as follows: 40 cycles of 30 s at 95℃, 1 min at 60℃and 30 s at 72℃. Two replicates were performed. Statistical analysis was performed using software SPSS 13.0, and the graph displayed expression levels of gene was drawn according to these data.

2.5 Analysis of putative transformants

The *PutSTE24* gene was amplified again from constructed cDNA library using primers associated *Bam*HI. The correctly modified gene was inserted into plant expression vector *pBI121* to construct *pBI121-PutSTE24*, and then the expression vector was introduced into Agrobacterium strain *EHA105* by electroporation method. Arabidopsis were transferred with this plant expression vector via *Agrobacterium tumefaciens*–mediated methods. The putative transgenic seeds (T1) were inoculated on 1/2 MS containing 50 mg/L Kana for resistance screening and detected with northern blot. The positive progeny were cultivated to T3 plants for stress experiment.

2.6 Over-expression of *PutSTE24* in transgenic Arabidopsis under aluminum stress

For testing the tolerance for Al stress, positive T2 plants (T2-4#, 5#, 6#) and untransformed (control, WT) plants were aseptically cultured in 1/2 MS included varies of $AlCl_3$ (0 μM, 200 μM, 300 μM, 400 μM) for 14 days. Three independent biological replicates were performed. The effects of phenotypic change and root length under Al stress were observed and recorded. These data were presented as the mean ± standard error from three independent experiments.

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