Author/s:
Geng, X;Horst, WJ;Golz, JF;Lee, JE;Ding, Z;Yang, ZB

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LEUNIG_HOMOLOG Transcriptional Co-repressor Mediates Aluminum Sensitivity through *PECTIN METHYLESTERASE46*-Modulated Root Cell Wall Pectin Methylesterification in Arabidopsis

Xiaoyu Geng[1], Walter J. Horst[2], John F.Golz[3], Joanne E. Lee[4], Zhaojun Ding[1]*, Zhong-Bao Yang[1]*

[1]The Key Laboratory of Plant Cell Engineering and Germplasm Innovation, Ministry of Education, School of Life Science, Shandong University, Jinan 250100, People's Republic of China
[2]Institute of Plant Nutrition, Leibniz Universität Hannover, Herrenhaeuser Str. 2, 30419 Hannover, Germany
[3]School of BioSciences, University of Melbourne, Victoria 3010, Australia
[4]Umeå Plant Science Centre, Department of Plant Physiology, Umeå University, SE-901 87 Umeå, Sweden

Corresponding author: Dr. Zhong-Bao Yang (zbyang@sdu.edu.cn) or Dr. Zhaojun Ding (dingzhaojun@sdu.edu.cn)

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SUMMARY

A major factor determining aluminum (Al) sensitivity in higher plants is the binding of Al to root cell walls. The Al binding capacity of cell walls is closely linked to the extent of pectin methylesterification, as presence of methyl groups attached to the pectin backbone reduces the net negative charge of this polymer and hence limits Al binding. Despite recent progress in understanding the molecular basis of Al resistance in a wide range of plants, it is not well understood how the methylation status of pectin is mediated in response to Al stress. Here we show in Arabidopsis that mutants lacking the gene *LEUNIG_HOMOLOG* (*LUH*), a member of the Groucho-like family of transcriptional corepressor, are less sensitive to Al-mediated repression of root growth. This phenotype is correlated with increased levels of methylated pectin in the cell walls of *luh* roots as well as altered expression of cell wall-related genes. Among the LUH-repressed genes, *PECTIN METHYLESTERASE46* (*PME46*) was identified as reducing Al binding to cell walls and hence alleviating Al-induced root growth inhibition by decreasing PME enzyme activity. *seuss-like2* (*slk2*) mutants responded to Al in a similar way as *luh* mutants suggesting a LUH-SLK2 complex represses the expression of *PME46*. The data are integrated into a model in which it is proposed that PME46 is a major inhibitor of pectin methylesterase activity within root cell walls.

INTRODUCTION

A major factor limiting crop production in acidic soils is aluminum (Al) toxicity. This arises when the pH of soil drops below 5 leading to increased solubility of Al and the formation of the trivalent cation Al^{3+} (Panda and Baluška, 2015). Al phytotoxicity is a worldwide problem, as it has been estimated that approximately 50% of the world's potential arable land is acidic (von Uexküll and Mutert, 1995). High levels of Al inhibit root growth (Horst et al., 1992; Delhaize and Ryan, 1995) leading to a damaged root system that limits nutrient and water uptake from soil, and thus impede plant growth (Kochian et al., 2004).

Plants have developed a wide variety of adaptive strategies to cope with Al toxicity in acid soils. There has been much recent progress towards understanding the physiological and molecular basis of Al resistance and tolerance in plants (Dehaize et al., 2012; Kochian et al., 2015), particularly in the model plant Arabidopsis. For instance, in response to Al stress, increased formation of the ALMT (Al-activated Malate Transporter) and MATE (Multidrug and Toxic Compound Extrusion) transporters enhances root exudation of malate and citrate, which chelates Al^{3+} and thus prevents its uptake by roots (Hoekenga et al., 2006; Kobayashi et al., 2007; Liu et al., 2009). Al stress is also associated with increased activity of the ABC transporter *ALS3* (Al-sensitive3), which is thought to redistribute toxic Al from Al-sensitive to non-sensitive root zones (Larsen et al., 2005), and *ALS1*, which may be responsible for the transport of Al into vacuoles where it is sequestered (Larsen et al., 2007). Central to many of these responses is the transcription factor *STOP1* (*Sensitive to Proton Rhizotoxicity 1*), which promotes expression of *ALMT1*, *MATE* and *ALS3* following Al exposure and thus plays a critical role in coordinating the transcriptional response to Al stress (Liu et al., 2009; Sawaki et al., 2009).

While the organic acid anions released from roots can limit Al entering into the root symplast, potential Al binding sites in the cell walls also compete with the organic acid anions for Al binding (Eticha et al., 2005). Composed of microfibrils embedded in a matrix of pectins, hemicelluoses and structural proteins, the primary cell wall either binds to Al^{3+} electrostatically, via the negatively charged carboxyl groups of wall pectins (mostly homogalacturonan; Mohnen, 2008), or via adsorption to uncharged hemicellulose polymers (Vose and Randall, 1962; Chang et al., 1999; Zhu et al., 2012). As a consequence of Al binding to the cell walls, loosening and anisotropic cell expansion in the elongation zone of the root is impeded (Jones et al., 2006; Kopittke et al., 2008; Rangel et al., 2009; Kopittke et al., 2014). The effect on root growth is rapid as demonstrated by a recent study showing that 75 μM Al^{3+}

reduces root elongation in soybean within 5 min due to Al binding to the rhizodermal and outer cortical cell walls (Kopittke et al., 2015). Short-term Al accumulation in roots is closely correlated to the pectin content in apical root sections, suggesting that Al bound to the pectic matrix determines Al sensitivity (Horst et al., 1999; Eticha et al., 2005; Yang et al., 2008; Rangel et al., 2009). It has been shown that Al sensitivity mainly depends on the degree of methylation of pectin, which is determined by pectin methylesterase (PME) activity (Micheli, 2001), as alterations to the methylation status of homogalacturonan (HG) affects the charge properties of the cell wall and hence its capacity to bind to Al (Eticha et al., 2005). For instance, higher Al accumulation in an Al-sensitive compared to an Al-resistant cultivar of maize was related to a lower degree of pectin methylation, while difference in pectin content were not apparent between these cultivars (Eticha et al., 2005). Furthermore, short-term PME treatment of intact maize roots, which decreases the extent of pectin methylation, enhanced both Al accumulation in root cells and Al-induced inhibition of root elongation (Horst et al., 2007). In potato (*Solanum tuberosum* L.), Al accumulation and Al injury quantified by callose production in the root tips and inhibition of root growth were associated with *PME* expression of transgenic plants (Schmohl et al., 2000, Horst et al., 2010). Similarly, cell wall PME activity and content of demethylated pectin in root tips were higher in Al-sensitive cultivars of rice (*Oryza sativa*) (Yang et al., 2008) and pea (*Pisum sativum*) (Li et al., 2016). Finally, transcriptional analysis of Al resistance in maize revealed that Al exposure caused significantly higher expression of a *PME* gene (MZ00000091) in Al-sensitive genotypes (Maron et al., 2008).

LEUNIG_HOMOLOG (*LUH*) and closely related *LEUNIG* (*LUG*) encode proteins belonging to the Groucho/TUP1 family of transcriptional corepressors (Liu and Karmarkar, 2008; Lee and Golz, 2012). Both are expressed broadly, and in many instances display functional redundancy. This is apparent during flower development, as well as during embryogenesis (Sitaraman et al., 2008). In other processes, such as enhanced resistance to abiotic stresses, LUH functions independently of LUG (Shrestha et al., 2014). Recent work has shown that LUH also modulates the pectin structure of both the Arabidopsis seed mucilage and primary cell walls of mucilage secreting cells of the testa (Western et al., 2001; Huang et al., 2011; Walker et al., 2011; Saez-Aguayo et al., 2013). Mucilage, which is released from the testa following contact with water, is primarily composed of the pectin rhamnogalacturonan I (RG-I) and to a much lesser extent HGs, hemicelluloses, and cellulose (Western et al., 2001). In contrast to mucilage, HG is typically the most abundant pectin polymer found in the primary cell walls of most higher plants, with RG-I constituting only a minor component of the pectin polymers (Cosgrove, 2005). *luh* mutant seeds exhibit a mucilage extrusion defect that arises from a failure of the mucilage to swell following contact of the seeds with water. This defect is correlated with increased presence of galactose residues attached to the RG-I backbone and reduced expression of the β-galactosidase *MUCILAGE MODIFIED 2* (*MUM2*) in the seed coat (Walker et al., 2011; Huang et al., 2011). In addition to alterative RG-I structure, elevated levels of methyl esterified HG are detected in both *luh* mucilage and the primary cell wall of mucilage secretion cells (Western et al., 2001; Walker et al., 2011). While reduced pectin methylesterase activity has been reported for *luh* mutant seeds, it does not arise from elevated activity of the main pectin methylesterase inhibitor PMEI6 present in Arabidopsis seeds (Saez-Aguayo et al., 2013).

Given the link between LUH and cell wall architecture in Arabidopsis, we investigated whether LUH plays a role in modulation of Al sensitivity in Arabidopsis roots. The results presented here suggest that LUH increases the capacity of cell walls to bind Al in response to Al stress. This function involves the repression of *PME46* (*PECTIN METHYLESTERASE 46*), a gene that apparently modulates the activity of pectin methylesterase within the cell wall.

RESULTS

luh mutants display enhanced resistance to Al stress

T-DNA insertion mutant lines *luh-3* (SALK_107245C) and *luh-4* (SALK_097509) with insertions at 4387 bp and 1453 bp from the start-ATG (Figure1a), respectively, were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Consistent with both conditioning strong mutant alleles, *LUH* expression was undetectable by

RT-PCR analysis (Figure 1b). To assess the response of these mutants to Al stress, we compared the extent of root growth inhibition of wild type and *luh* mutants on phytogel-solidified medium or in hydroponics following exposure to different concentrations of Al. While increasing Al concentrations gradually inhibited root growth of both wild type seedlings and *luh-3* and *luh-4* mutants, *luh-3* and *luh-4* showed significantly less inhibition (Figure 1c-e). This indicates that *LUH* is a negative regulator of Al resistance. No differences in root growth were observed when wild type and *luh* mutants plants were exposed to different pH solutions or metal ions other than Al^{3+} (Figure 1f, g), suggesting a specific role for *LUH* in modulating responses to Al.

To know whether Al affects the expression of *LUH*, we first treated hydroponically grown *pLUH:GUS* transgenic lines (Stahle et al., 2009) with Al. Short-term Al exposure for 6 and 24 h, was associated with reduced GUS activity in the root tips of these lines (Figure S1a). This observation was subsequently confirmed by qRT-PCR analysis which showed that *LUH* expression was reduced by ~30% (Figure S1b). While statistically significant, this reduction is clearly not sufficient to alleviate the sensitivity of wild type roots to Al stress.

LUH promotes Al accumulation in the cell wall of roots

Hematoxylin staining of Al distribution in root tips of *luh-3* and *luh-4* mutants under Al stress revealed reduced Al accumulation in comparison to wild type roots (Figure 2a). Consistent with this observation, quantitative analysis of Al in 5-mm root tips and whole intact roots showed that *luh* mutants had significantly lower Al contents than wild type roots (Figure 2b, c). Subsequent fractional analysis of cellular Al distribution revealed that the reduced Al content in *luh* roots mainly resulted from significantly less Al accumulation in the cell wall rather than the symplast (Figure 2d). Furthermore, *in vitro* analysis of Al binding to ethanol-isolated cell walls showed lower binding capacity in *luh* root cell walls compared to wild-type (Figure 2e). Taken together, these results demonstrate that LUH promotes the binding capacity of root cell walls to Al.

LUH-suppressed pectin methylation increases Al binding to cell wall

LUH has been reported to regulate the methylation of homogalacturonan (HG) in both the mucilage and primary cell walls of the mucilage secreting cells of the seed coat (Western et al., 2001; Walker et al., 2011). In these and other studies, differences in methylation of HG polymers have been visualized using the monoclonal antibodies JIM5 (staining sparsely methylated pectins) and JIM7 (staining highly methylated) (Eticha et al., 2005; Walker et al., 2011). Application of these antibodies to roots revealed reduced JIM5 and elevated JIM7 fluorescence signals in the *luh* mutants compared to the WT. This is consistent with increased methyl esterification of HG residues in these lines (Figure 3a-c). Following Al treatment, the JIM5 fluorescence signal was significantly enhanced (Figure 3a, left panels; Figure 3b) whereas the JIM7 signal was reduced (Figure 3a, right panels; Figure 3c) in root tips of both wild type seedlings and *luh* mutants. This shows that Al treatment reduces pectin methylation independent of *LUH* expression.

Aluminum exposure did not significantly alter the pectin content of roots of wild type and *luh* mutants (Figure 3d). However, the degree of pectin methylation was significantly reduced in the wild type but not the *luh* mutants (Figure 3e), leading to an increased amount of unmethylated pectin in the wild type but not the *luh* mutants (Figure 3f). Compared to the wild type, both *luh* mutant roots had slightly lower pectin contents, but an increased degree of methylation (Fig. 3d) and thus lower content of unmethylated pectin (Figure 3f). Al slight decreased the content of methylated pectin in roots of *luh* mutants (Figure 3e, f); but the differences were not as great as that seen by immunofluroscence (Figure 3a-c). This difference likely reflect more severe Al stress in the root tips which were used for the immuno-microscopy compared to the whole roots used for the analysis of the pectin content. Overall these results clearly show that *luh* mutants have lower levels of unmethylated pectin in their cell walls (Figure 3f) leading to reduced Al binding in the root cell walls and thus less Al injury.

Transcriptome profiling of *LUH*-mediated Al resistance

To investigate the role of *LUH* in Al-induced inhibition of root growth and Al binding to cell walls, we examined the transcriptional profile of wild type and *luh-4* mutant roots in response to the presence or absence of Al using RNA-seq (Data S1). This analysis revealed that 652 genes in wild type (316 elevated, 336 reduced) and 544 genes in *luh-4* mutant roots (239 elevated and 305 reduced) displayed a two-fold or greater difference in expression following exposure to Al. Subsequent comparisons identified 141 genes (103 genes elevated, 38 genes reduced) that were differentially expressed in untreated *luh* mutants roots compared to wild-type, whereas 110 genes (77 genes elevated, 33 genes reduced) were differentially expressed in Al-treated *luh*-4 roots compared to Al-treated wild type roots (Figure S2). According to a GO analysis the *LUH*-dependent 110 differentially expressed genes in response to Al stress could be classified into a diverse range of categories (Figure 4a, b; Data S2): 37 (33.6%) genes associated with cell wall organization/biogenesis (30, up; 7, down),19 (17.3%) genes in unknown/unclassified (13, up; 6, down), 17 (15.5%) genes in stress/defense (8, up; 9, down), 10 (9.1%) genes in metabolic process (6, up; 4, down), 8 (7.3%) genes in transport (5, up; 3, down), 7 (6.4%) genes in cellular protein modification (5, up; 2, down), 6 (5.5%) genes in signal transduction (5, up; 1, down). Thus cell wall modification appears to be a major response to Al exposure in the *luh* mutant lines.

The transcription of twelve of differentially transcribed genes with putative function in cell wall organization were randomly selected and characterized using qRT-PCR. This analysis confirmed the results obtained from RNA-seq experiments (Figure 4c). Further, statistically analysis revealed a highly significant correlation between the RNA-seq and qRT-PCR data (Figure S2b). As PMEs play key roles in the regulation of cell wall pectin methylation (Micheli, 2001), it is possible that altered expression of these genes might account for enhanced resistance against Al stress in *luh* mutant roots. Mining the RNA-seq data for PME associated genes revealed reduced expression *PME17* (AT2G45220) and one member of the PMEI family (AT3G 17130) in *luh-4* mutant roots, but increased expression of *PME46* (AT5G04960) in the absence of Al. Furthermore, exposure to Al stress reduced expression of *PME17*, *PME54* (AT5G20860), *PME46* and two members of the PMEI family *AT3G17130* and *AT4G25250*in both wild type and mutant. The repression by Al of *PME54* and *AT3G17130* was greater in the *luh-4* mutant compared to the wild type, but lower for *PME46* and *AT4G25250* (Figure S3). Reduced expression of *PME* and *PMEI* genes following Al exposure is surprising given that PME activity is predicted to increase in response to Al stress. Similarly, increased expression of *PME46* in the *luh-4* mutant is unexpected as PME activity in this mutant line is predicted to be lower than in the wild type. qRT-PCR analysis subsequently confirmed that *PME46* expression was higher in *luh* mutant roots in comparison to the wild type (Figure 4c). This is consistent with a negative regulation of PME46 by LUH. Since *ALMT1*, *MATE*, *STOP1*, *ALS1* and *ALS3* genes are associated with Al resistance in Arabidopsis (see Introduction), we analysed their expression. Our analysis failed to identify differences in the expression of these genes in *luh* mutant roots compared to wild type under Al stress (Figure S4).

PME46 mediates Al binding to cell wall and thus Al sensitivity

To investigate whether *PME46* might be associated with the different responses of wild type and *luh* mutant roots to Al, two T-DNA insert mutants *pme46-1* (SAIL_612_D02) and *pme46-2* (SALK_136669) were obtained from NASC (Figure S5a). qRT-PCR analysis confirmed that both mutants had reduced *PME46* expression compared to wild-type (Figure S5b). Al stress reduced root growth in both wild type and *pme46-1* and *pme46-2* mutants. However, the degree of inhibition was greater in the mutants compared to wild type roots (Figure 5a, b). Hematoxylin staining of Al distribution in root tips revealed a greater Al accumulation in the *pme46* mutant lines compared to the wild type (Figure 5c), which was further confirmed when analyzing the Al content in whole roots (Figure 5d). Fractional analysis of cellular Al distribution showed that the elevated Al content in roots of the *pme46* mutants is mainly associated with the cell wall rather than the symplast (Figure 5e). As increased Al binding to cell walls is associated with reduced pectin methylation, we determined the levels of PME activity. The analysis revealed that the PME enzyme activity was elevated in the roots of the *pme46* mutants compared to the wild type (Figure 5f), suggesting that *PME46* represses PME enzyme activity. Based on these observations, we propose that elevated levels of *PME46* expression in *luh* mutant roots account for the reduced PME activity observed in this line.

***SLK2* functions similar with LUH to promote Al binding to the root cell wall**

SEUSS (SEU) and SEUSS-LIKE2 (SLK2) can physically interact with LUH forming SEU/SLK2-LUH co-repressor complexes in yeast assays (Sitaraman et al., 2008; Stahle et al., 2009; Shrestha et al., 2014) and *in planta* (Lee et al., 2014). While *seu* mutant did not differ from the wild type in Al sensitivity, both *slk2-1* and *slk2-2* mutant roots displayed higher levels of Al resistance, with *slk2-1* being noticeable more resistant than *slk2-2* (Figure 6a). Despite increased Al resistance seen in *slk2-2* mutants, hematoxylin staining in root tips and Al content in whole intact roots was only reduced in *slk2-1* compared to wild-type (Figure 6b, c), and this reduction mainly resulted from a lowered Al accumulation in cell walls (Figure 6d). The observed differences between *slk2-1* and *slk2-2* mutants likely reflect the fact that *slk2-1* is a near null mutant allele, whereas *slk2-2* was not an RNA null allele and hence predicted to be hypomorphic (Bao et al., 2010). Similar to *LUH*, *SLK2* promoter activity was detected in root tips and reduced following exposure to Al stress (Figure S6). In addition, expression of *PME46* was elevated in *slk2-1* mutant roots (Figure 6e) which were less inhibited in root growth by Al (Figure 6a).

DISCUSSION

Although substantial physiological evidence suggests that pectin plays a crucial role in determining the capacity of the root cell walls to bind Al and hence mediate Al resistance (Horst et al., 2010), the molecular pathways involved in this response are poorly understood. The work reported here identifies a link between the cell wall pectin defect of Arabidopsis *luh* mutant roots and resistance to Al-induced root growth inhibition (Figure 1). While the pectin defects in the seed coat mucilage of *luh* mutants mainly involve changes to RG-I structure, brought about by reduced MUM2 activity, there is also evidence of increased HG esterification in the cell wall of the mucilage secreting cells of the *luh* testa (Western et al., 2001, Walker et al., 2009; Huang et al., 2009). Given the known link between HG esterification and Al sensitivity, it is likely that the increased Al resistance observed in *luh* roots is due to elevated esterification of HG in the walls of these cells. This is because elevated methylation of HG reduces its overall charge, and hence limits the number of potential binding sites for Al in cell walls (Eticha et al., 2005). It is less likely that changes to RG-I structure account for the altered Al binding properties of *luh* roots. This is principally due to RG-I being a minor component of plant cell wall pectins, no established link between RG-I and Al binding and the fact that *mum2* mutant roots display the same sensitivity to Al as wild type roots (Figure S7).

Like LUG, LUH lacks a DNA binding domain and is therefore recruited to the regulatory sequences of target genes either through direct association with transcription factors, or indirectly, via the coregulator proteins SEUSS (SEU) and SEUSS-LIKE (SLK) (Sridhar et al., 2004; Gregis et al., 2006; Sridhar et al., 2006; Stahle et al., 2009; Grigorova et al., 2011; Shrestha et al., 2014; Lee et al., 2015). While mutations in the SEU/SLK coregulators phenocopy *lug* and *luh* mutants to some extent, there is often variation between mutants (Bao et al., 2010; Shrestha et al., 2014). Indeed this was reflected in our study, where *slk2* but not *seu* mutants, displayed increased resistance to Al-induced root-growth inhibition (Figure 6a). While this difference might reflect functional divergence between the SEU and SLK2 proteins, a more likely explanation is that these genes have different transcriptional responses to Al stress. Regardless, our data clearly shows that LUH and SLK2 are both involved in Al stress resistance, and hence consistent with the formation of a LUH-SLK2 complex in roots. Loss of SLK2 is also correlated with reduced expression of *PME46*, suggesting that the LUH and SLK2 share a common downstream target.

Pectins are methylated in medial-Golgi and secreted as highly methylated form into cell walls, where they are subsequently demethylated by PMEs (Micheli, 2001).

Several studies have demonstrated that Al exposure enhances PME enzyme activity, which reduces the degree of methylation associated with pectins, and thus creating more Al binding sites in the cell wall (Schmohl et al., 2000; Yang et al., 2008; Li et al., 2016). For instance, maize plant roots treated with PME (Schmohl et al., 2000), or rice plants overexpressing *PME* genes (Yang et al., 2013) bind more Al to cell walls and display a greater Al sensitivity. *PME*s are part of a large multigene family in land plants (Markovic and Janecek, 2004; Yokoyama and Nishitani, 2004), which in Arabidopsis consists of 66 members (Sénéchal et al., 2015). *PME46* (AT5G04960), a potential regulator of PME enzyme activity, is classified as a member of type I/group 2 PME (proPME) subfamily that has an N-terminal pro region with extensive similarity to the PME inhibitor (PMEI) domain of *PMEI* genes (Micheli, 2001; Pelloux et al., 2007; Wang et al., 2013). The pro region enables retention of unprocessed PMEs in the Golgi apparatus which limits their activity and thus forms part of a post-translational regulatory mechanism (Wolf et al., 2009; Sénéchal et al., 2014). Similarly, a study in *Nicotiana tabacum* has shown that the pro region of a pollen-specific PME (NtPPME1) acts as an intracellular inhibitor of PME activity to prevent premature demethylation of pectins (Bosch et al., 2005). While it is possible that the pro domain of PME46 might inhibit the activity of other PMEs. Indeed, a recent study has shown that PMEI7 represses PME3 activity through interaction with a PME ligand-binding cleft structure (Sénéchal et al., 2015). An alternative possibility is that elevated PME46 activity triggers transcriptional repression of other *PMEs*, thus the precise mechanism remains to be elucidated, but is supported by the following observations: (1) *pme46* mutants have increased PME activity compared to the wild type, (2) *pme46* mutants accumulate higher levels of Al in the cell wall of roots, (3) *pme46* mutants are more sensitive to Al stress (Figure 5a-f), and (4) *PME46* shows similar transcriptional response as PMEI *AT4G25250* (Figure S3).

In conclusion, we propose that the increased resistance to Al stress in *luh* and *slk2* mutants likely reflects a combination of factors. The first is associated with an increase in the amount of methylated pectin located in the cell wall of mutant root prior to Al stress. This change presumably reflects reduced PME activity, which has been reported in the seed coat of *luh* mutants (Saez-Aguayo et al., 2013). While increased *PMEI* expression might account for the altered PME activity observed in *luh* roots, this possibility seems unlikely given that changes to *PMEI* expression were not observed in our transcriptomic analysis (Data S1). A similar observation has been made in the *luh* mutant seed coat, where increased PME activity is actually correlated with reduced expression of the main PME found in this tissue (Saez-Aguayo et al., 2013). Instead, we propose that elevated *PME46* expression is the causal factor leading to reduced PME activity. And, as a consequence, cell walls of *luh* and *slk2* mutants are composed of less charged HG, which bind less Al and thus protect the roots from Al stress.

The second factor accounting for increased resistance of *luh* and *slk2* roots to Al stress relates to the changes in cell wall composition that occur following Al exposure. In wild type, Al stress is associated with reduced esterification of pectin (presumable due to elevated PME activity) and increased capacity of the cell wall to bind Al. These changes correlate with decreased *PME46* expression, which is predicted to release PMEs from repression. In contrast, *PME46* expression in *luh* and *slk2* roots is not reduced to levels seen in wild type roots following Al exposure, and there is only a marginal decrease in pectin methylation. As a result, Al exposure does not increase the capacity of *luh* roots to bind Al, which presumably accounts for Al resistant phenotype exhibited by these mutants.

These results can be integrated into a model that describes how Al affects the methylation status of pectin in the root cell walls (Figure 7). According to this model, *PME46* is a major inhibitor of PME activity and is repressed by the LUH/SLK2 complex. A prediction arising from the model is that in *luh* and *slk2* mutants, *PME46* is de-repressed, which reduces PME activity, allowing methylated pectin to accumulate in the cell walls. As these pectins have a lower negative charge, they will bind less Al, which accounts for the Al resistance phenotype of *luh* and *slk2* mutants. Exposure of roots to Al activates a second pathway involved in *PME46* repression (Figure 7). Reduced *PME46* expression allows PMEs to become more active, which reduces the esterified pectin content of cell walls. The increased accumulation of negatively charged pectins in the cell wall provides sites for Al binding and hence increased Al sensitivity of wild type roots.

The study provides a framework for understanding how cell wall modification pathways are activated in response to Al stress. As illustrated in Figure 7, these pathways are complex with only a few of the components identified. It is not known whether the LUH/SLK2 complex is directly recruited to the regulatory elements of *PME46*. Also the transcription factors that are involved in this process need to be identified. Similarly, the predicted Al-induced stress response pathway that mediates repression of *PME46* independently of LUH is currently not defined. No doubt, future studies will need to address these issues in order to provide a more complete picture of how plants respond to Al stress.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions

Arabidopsis mutants *luh-3* (SALK_107245C), *luh-4* (SALK_097509), *seu-4* (SALK_069303, Pfluger and Zambryski, 2004), *slk2-1* (SALK_089954, Alonso et al., 2003), *slk2-2* (SALK_089954, Alonso et al., 2003), *mum2-10* (CS16346), *mum2-11* (SALK_110461), *pme46-1* (SAIL_612_D02), and *pme46-2* (SALK_136669), transgenic lines *pLUH:GUS* (Stahle et al., 2009) were used. Mutants *luh-3*, *luh-4*, *pme46-1* and *pme46-2* were obtained from the Nottingham Arabidopsis Stock Centre (NASC).

The seedlings were grown either on solidified gel medium according to Larsen et al. (1996) or in hydroponics. For the soaked gel plates, the nutrient medium (pH 4.2) consisted of 40 ml of 3 mM $MgCl_2$, 0.25 mM $(NH_4)_2SO_4$, 2 mM KCl, 1 mM $Ca(NO_3)_2$, 2.75 mM $CaCl_2$, 0.18 mM KH_2PO_4, 100 µM $MnSO_4$, 500 µM H_3BO_3, 20 µM $ZnSO_4$, 5 µM $CuSO_4$, 2 µM $NaMo_7O_{24}$, 0.1 µM $CoCl_2$, 1% sucrose, and 0.3% Gellan gum (Sigma, Aldrich). For Al treatments, the solidified nutrient medium was soaked with 30 ml of various $AlCl_3$ concentration (pH 4.2) for 48 h. Afterwards, the soaking solution was discarded, seeds were sown and geminated for 2 d at 4 ^{o}C and further grown for 7 d in a growth chamber. For the dose-response analysis of root growth in hydroponics, seeds were geminated on plastic mesh floating onto the nutrient solution containing $AlCl_3$ (pH 4.2) and the same nutrients as described above for 7 d or nutrient solution without $AlCl_3$ for 6 d and then were transformed to the various concentration of $AlCl_3$ treatment. All growth analysis was performed in a growth chamber with a 16/8-h light/dark cycle at 22°C.

RNA Isolation and qRT-PCR

Approximately five hundreds six-day old seedlings were treated with $AlCl_3$, and shock frozen in liquid nitrogen after harvest. Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer’s protocol, and first-strand cDNA was synthesized from 1 µg of total RNA using the Transcript or First Strand cDNA Synthesis Kit (Roche) following the manufacturer’s protocol. qRT-PCR was performed using the CFX Connect Real-Time System (Bio-Rad) with Fast Start Universal SYBR Green Master (Rox) (Roche). Samples for qRT-PCR were run in three biological replicates and two technical replicates. For the normalization of gene expression, the ubiquitin gene *UBQ1* (AT3G52590) was used as an internal standard, and the non-treated wild type was used as a sample control. Primers were designed using Primer 5 software, and the specifications of the primers of the genes studied are given in Table S1.

RNAseq Analysis

Approximately five hundreds seedlings (6-day-old) of both the wild type Columbia and *luh-4* mutant line were exposed to 0 or 25 µM $AlCl_3$ (pH 4.2) in hydroponics. After 4 h, roots were sampled and RNA was isolated. RNAseq analysis was performed by BGI Tech (Shenzhen, China) via Ion Proton platform. The detailed technical and data analysis were performed as described by Yang et al. (2014) and available on request.

Generation of transgenic construct

To generate *pSLK2::GUS*, the 1.8-kb *SLK2* promoter was amplified from genomic DNA using primers pSLK2-F1 (5’-AACTCGAGCAAAGAGAAGTAAATACAC-3’) and pSLK2-R1 (5’-TTGGTACCCAAGTGAGTCTGAAATCC-3’) and cloned into the *Xho*I/*Kpn*I sites of the GUS containing shuttle vector pRITA. The *pSLK2::GUS* cassette was then placed in the binary vector pMLBART before being transformed into the GV3101 strain of *Agrobacterium tumefaciens* by electroporation. The construct was introduced in Col-0 plants by floral dipping and T2 plants histochemically stained for GUS activity. A representative line was subsequently selected for further experimentation.

GUS staining

For histochemical analysis of GUS activity, the seedlings were immersed into the staining solution consisting of 0.1 M sodium phosphate buffer (pH 7.0), 2 mM potassium ferri- and ferrocyanide, 0.1% Triton X-100) and 2 mM X-glucuronide at 37°C overnight. The samples were observed and photographed with an Olympus BX53 microscope equipped with an Olympus DP72 camera system (Japan).

Isolation of cell wall material and cell wall binding capacity of Al

Aproximately 10 mg seeds of wild type and *luh* mutants in dry weight were geminated on the plastic mesh floating onto the nutrient solution (pH 5.5) for 6 days, then the whole intact roots were excised and the cell wall material were isolated according to Yang et al. (2010). The isolated cell wall materials were incubated for 30 min in 1 ml of a solution (pH 4.2) containing 600 μM $AlCl_3$. Then the suspension were centrifuged at 23 000 g for 10 min and the supernatant was discarded. The pellet was washed with ultra-pure deionized water for twice. The residues were prepared for Al determination.

Localization of pectin by immunofluorescence

Localization of pectin by indirect immunoflorescence was performed using monoclonal antibodies which are specific for pectin with different degrees of methylation according to Eticha et al. (2005). Roots were collected into a fixative solution containing 4% paraformaldehyde in 50 mM 1,4-piperazine-diethanesulphonic acid (PIPES), 5 mM $MgSO_4$, and 5 mM ethylene glycol bis (β-amino-ethylether)-N,N,N′,N′-tetraacetic acid (EGTA), pH 6.9, for 1-2 h, and then the samples were washed repeatedly with phosphate-bufferedsaline (PBS) and blocked with 0.2% BSA in PBS buffer for 30 min. Then the samples were incubated in diluted solution of primary antibodies (JIM5 and JIM7) for 2 h at room temperature. The primary antibody was thoroughly washed off the samples with PBS three times for 5 min, each. Next, they were incubated for 2 h in the 50-fold diluted solution of the secondary antibody, abtirate-IgG coupled with fluorescein isothiocyanate (FITC). The samples were washed as mentioned above, and mounted on glass slides and examined underan LSM-700 laser-scanning confocal microscope (Zeiss, Germany).

Cell wall pectin content and degree of pectin methylation

Cell wall pectin content and degree of pectin methylation were performed according to Eticha et al. (2005). The alcohol-insoluble cell wall materials of roots were first isolated with 96% ethanol, weighed and hydrolysed by incubating samples in concentrated H_2SO_4. The uronic acid content was determined colorimetrically using a microplate spectrophotometer (Infinite 200 PRO, Tecan, Switzerland). Galacturonic acid was used as a calibration standard, thus the root pectin content was expressed as galacturonic acid equivalents (GaE).

The degree of pectin methylation was calculated according to the amount of released methanol from cell wall material. After addition of 2 U alcohol oxidase (from Piccia pastoris, Sigma), the complex of formaldehyde with Fluoral-P (15 mg ml^{-1}) (Sigma) was measured fluorometrically.

Root cell sap preparation and Al determination

After treatment, roots were washed at least for three times with double distilled water, excised, transferred into a 0.45 μm unit of centrifugal filter (PALL, USA) and then centrifuged at 3,000 g for 10 min at 4^{o}C to remove apoplastic solution. The roots were then frozen at -80^{o}C overnight. The root cell sap was obtained by thawing the samples at room temperature and then centrifuging at 22,000 g for 10min. The pellet was washed with 70% (v/v) ethanol for three times and designated as the cell wall fraction. For the determination of Al in roots or root fractions, the samples were digested with concentrated 65% ultra-pure HNO_3, and after approximate dilution, the Al concentration was determined by GFS-AAS (SHIMADZU, Japan).

PME enzyme activity assay

For the extraction of PME, roots were homogenized and suspended in the extraction buffer containing 100 mM Tris-HCl (pH 7.5) and 1M NaCl. Extracts were centrifuged at 23, 000g for 10 min and the supernatant were collected. PME activity was determined according to Anthon and Barrett (2004). Fifty microliters of PME extract were added into a reaction mix containing 100 mM PBS buffer, 0.4 mg ml^{-1} pectin (Sigma), 0.1 U alcohol oxidase, and incubated at $30^{o}C$ for 10 min. Then 200 μl of 0.5 M NaOH solution containing 5 mg ml^{-1} Purpald was added. After incubation at $30^{o}C$ for 30 min, 600 μl distilled water was added to give a final volume of 1 ml. The absorbance at 550 nm was measured with a spectrophotometer (Infinite 200 PRO, Tecan, Switzerland).

Statistical Analysis

Statistical analysis was performed using SAS 9.2 (SAS Institute). Means were compared using Student's *t* test. For the statistical analysis in root growth experiments, three biological replicates for each treatment were performed. For each replicate, the mean of fifteen uniform primary roots was used. Asterisks in the figures denote significant differences as follows: $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

ACCESSION NUMBERS

Sequence data from this article can be found in the Arabidopsis Genome Initiative database and the GenBank/EMBL databases under the following accession numbers: *LUH* (AT2G32700, NM_128829), *PME46* (AT5G04960, NM_120578), *SEU* (AT1G43850, NM_103511), *SLK2* (AT5G62090, NM_125602), *ALMT1* (AT1G08430, NM_100716),*MATE* (At1g51340, NM_104012), *STOP1* (AT1G34370, NM_103160),

ALS3 (AT2G37330, NM_129289), *ALS1* (AT5G39040, NM_123266). RNA sequencing data analyzed in this study are available in the Gene Expression Omnibus database under accession number GSE85292.

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SHORT LEGENDS FOR SUPPORTING INFORMATION

Figure S1. LUH expression is responsive to Al treatment.

Figure S2. Transcriptional profiling of *LUH*-regulated genes in response to Al stress.

Figure S3. Expression of *PMEs* in wild type and *luh-4* mutant in response to Al stress based on RNA-seq.

Figure S4. Expression of Al-resistant related genes in response to Al stress.

Figure S5. *PME46* gene expression in wild type seedlings and *pme46* T-DNA insert mutants.

Figure S6. Expression of *SLK2* in roots exposed to Al stress.

Figure S7. No root growth difference between *mum2* mutant and wild type plants in response to Al stress.

Table S1. List of genes and specific primer pairs used for quantitative real-time PCR (qRT-PCR).

Data S1. Comparison of the differentially expressed genes in roots of wild type and *luh-4* exposed to Al stress.

Data S2. Up- and Down-regulated genes in roots of Al-exposed *luh-4* mutant.

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FIGURE LEGENDS

Figure 1. *luh* mutants display enhanced resistance to Al stress. (a) Schematic representation of T-DNA insertions within the *LUH* locus. (b) Expression of *LUH* in wild type (WT) seedlings and mutants *luh-3* and *luh-4*. (c-e) Root growth analysis

under Al stress. WT seedlings and mutant were grown on gel medium containing 0 to 1.5 mM $AlCl_3$ (c), or in hydroponics containing 0 to 40 μM $AlCl_3$ (d and e), at pH 4.2 for 7 days. (f) Root growth response of WT seedlings and mutant lines *luh-3* and *luh-4* grown in solutions with pH 5.8, pH 5.0 and pH 4.2 for seven days. (g) Root growth responses of WT and *luh* mutants grown on gel-solidified media containing 1.25 mM $AlCl_3$, or 5 μM $CuCl_2$, or 25 μM $CdCl_2$, or 100 μM $LaCl_3$ for 7 days. In (c, e-g): *, **, and *** indicate a significant difference between WT seedlings and mutant lines at $p < 0.05$, $p < 0.01$, and $p < 0.001$ (*t*-test), respectively. Values shown are means ± SD (n = 3).

Figure 2. LUH mediates Al accumulation in the cell wall of roots. (a) Hematoxylin staining of root tips exposed to Al. (b) Al content in 5-mm root tips and (c) whole intact roots of wild type (WT) seedlings and mutants. (d) Fractional analysis of cellular Al distribution in cell wall and symplast of Al-exposed roots of WT seedlings and mutants. (e) Al binding capacity of cell wall in roots of WT seedlings and mutants. In (b-d): Six-day old WT seedlings and mutants *luh-3* and *luh-4* were treated with 25 μM $AlCl_3$ for 24 h. In (e): Roots of the six-day old WT seedlings and *luh* mutants were harvested andcell wall material isolated. Isolated cell wall material was treated with 1 ml 600 μM $AlCl_3$ for 30 min at pH 4.2.Values shown are means ± SD (n = 4). * and ** indicate significant differences between WT and mutant at $p < 0.05$ and $p < 0.01$ (*t*-test), respectively.

Figure 3. *LUH*-suppressed pectin methylation mediates Al binding to cell wall. (a) JIM5 and JIM7 staining to detect the low- and high-methylated pectin in cell wall of wild type (WT) and mutants. Bar = 100 μm. (b and c) Quantitative analysis of the fluorescence intensity of JIM5 staining (b) and JIM7 staining (c) indicated in (a) in the root tips. Values are means ± SD (*n* = 6). (d) Pectin content, (e) degree of pectin methylation, and (f) unmethylated pectin content in roots of WT plants and mutants under Al stress. In (a-f) six-day old seedlings were exposed to 25 μM $AlCl_3$ for 24 h. Values in (d-f) represent means ± SD (n = 4). *, **, and *** indicate significant

differences at $p < 0.05$, $p < 0.01$, and $p < 0.001$ (*t*-test), respectively, between either –Al and +Al treatments (in black) or WT and mutant (in red).

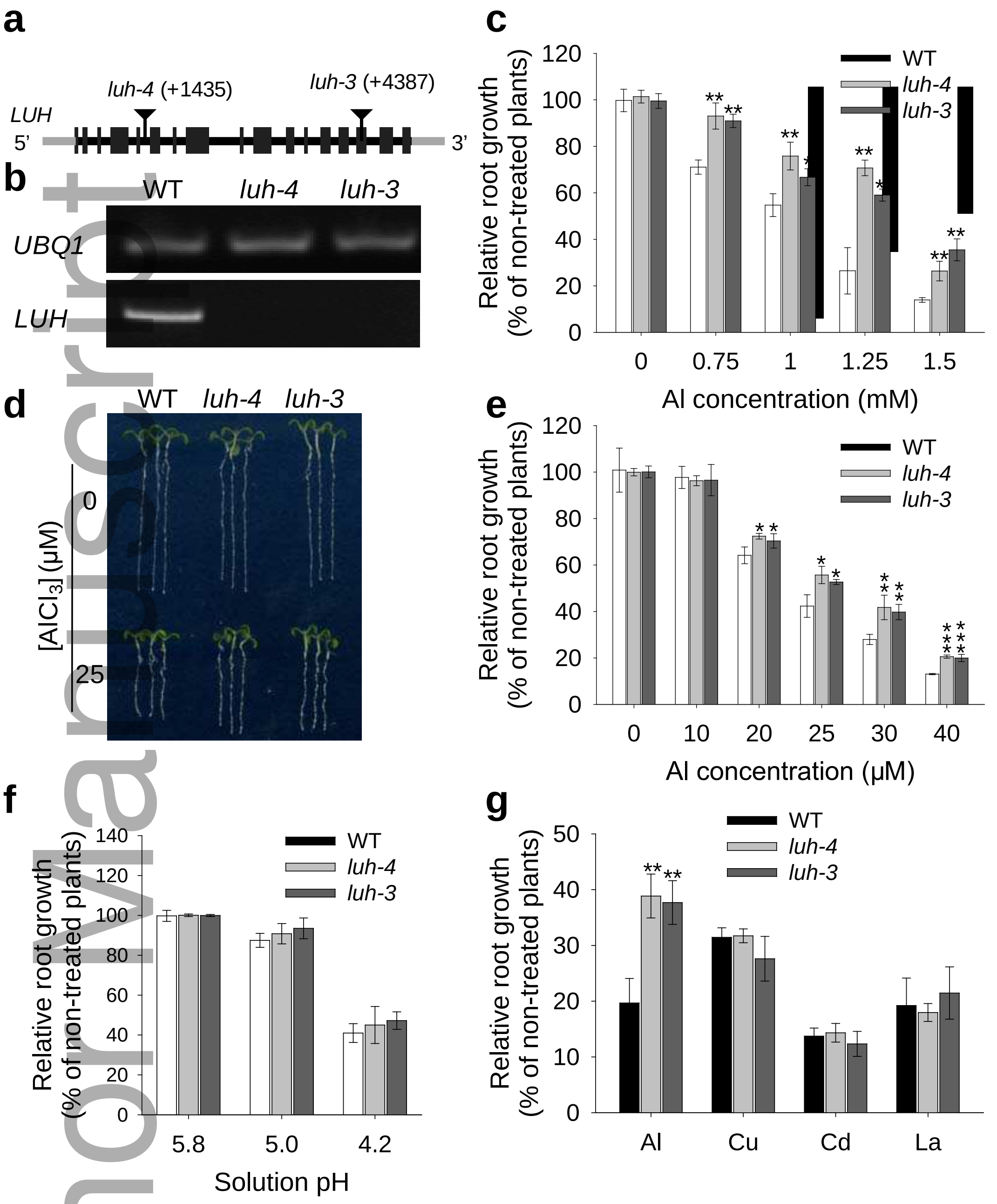
Figure 4. Transcriptome analysis of *LUH*-regulated genes in response to Al stress. (a) Functional categorization of differentially transcribed genes with 2-fold changes or more in response to Al stress. (b) Cluster analysis of the expressed genes with more than two-fold changes in wild type (WT) and *luh-4* mutant seedlings in response to Al stress. The cluster analysis of cell wall (CW)-associated genes was particularly listed. RPKM (Reads Per kb per Million reads) represents the gene expression level. (c) Validation of the expression of several selected CW-associated genes listed in (b) by qRT-PCR. Six-day-old WT and *luh-4* seedlings were exposed to 0 and 25 µM $AlCl_3$ for 4 h. *UBQ1* was used as the reference, and a non-treated WT was used as the sample control. Values represent means ± SD (n = 3). Asterisks in black indicate that wild-type and *luh-4* mutant and asterisks in red indicate that Al treatment means differ significantly at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (*t*-test), respectively.

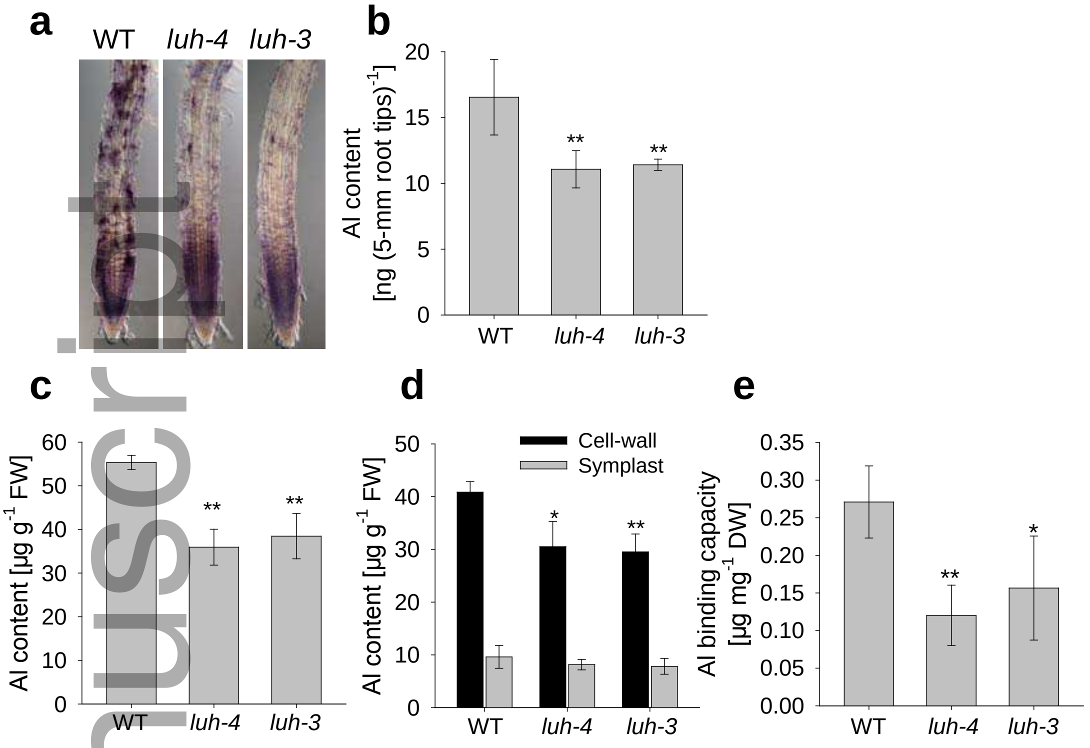
Figure 5. *PME46* mediates Al-induced inhibition of root growth and Al binding to cell wall. (a, b) Root growth of wild type (WT) plants, *pme46-1* and *pme46-2* mutant lines under Al stress. WT seedlings and mutant were grown into the hydroponic solution containing 0 to 25 µM $AlCl_3$ at pH 4.2 for 7 days. Means represent values ± SD (n = 3). (c) Hematoxylin staining of Al distribution in root tips. (d) Al content in roots of WT seedlings and mutants. (e) Fractional analysis of cellular Al distribution in cell wall and symplast of Al-subjected roots of WT seedlings and mutants. (f) PME enzyme activity in roots of WT seedlings and mutants. In (c-f): Six-day old WT seedlings and mutants were treated with 25 µM $AlCl_3$ for 24 h. Values in (d-f) represent means ± SD (n = 4). *, ** and *** indicate the significant difference between WT and mutant at $p < 0.05$, $p < 0.01$, and $p < 0.001$ (*t*-test), respectively.

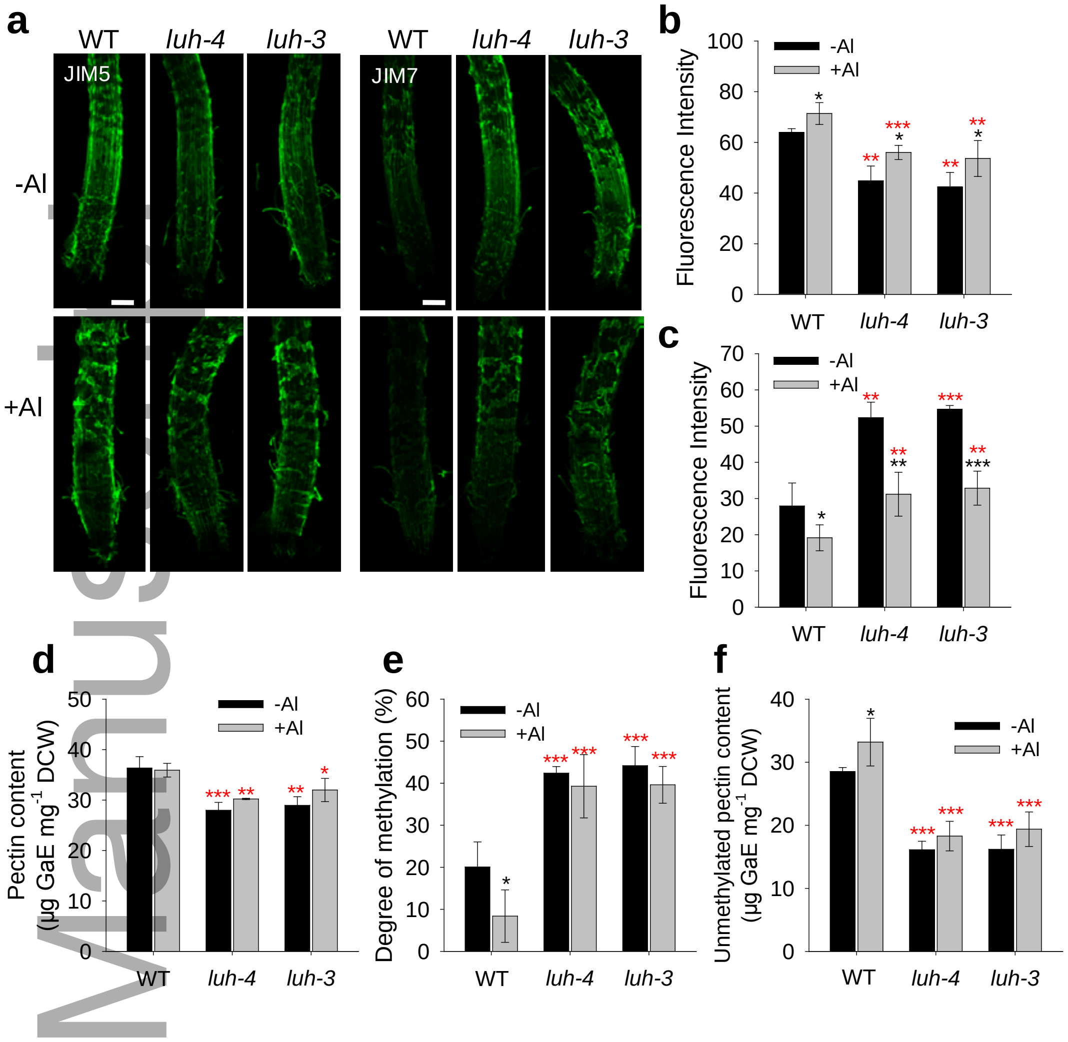
Figure 6. *SLK2* mediates Al-induced inhibition of root growth and Al binding to cell walls. (a) Root growth of wild type (WT) plants and mutants under Al stress. WT

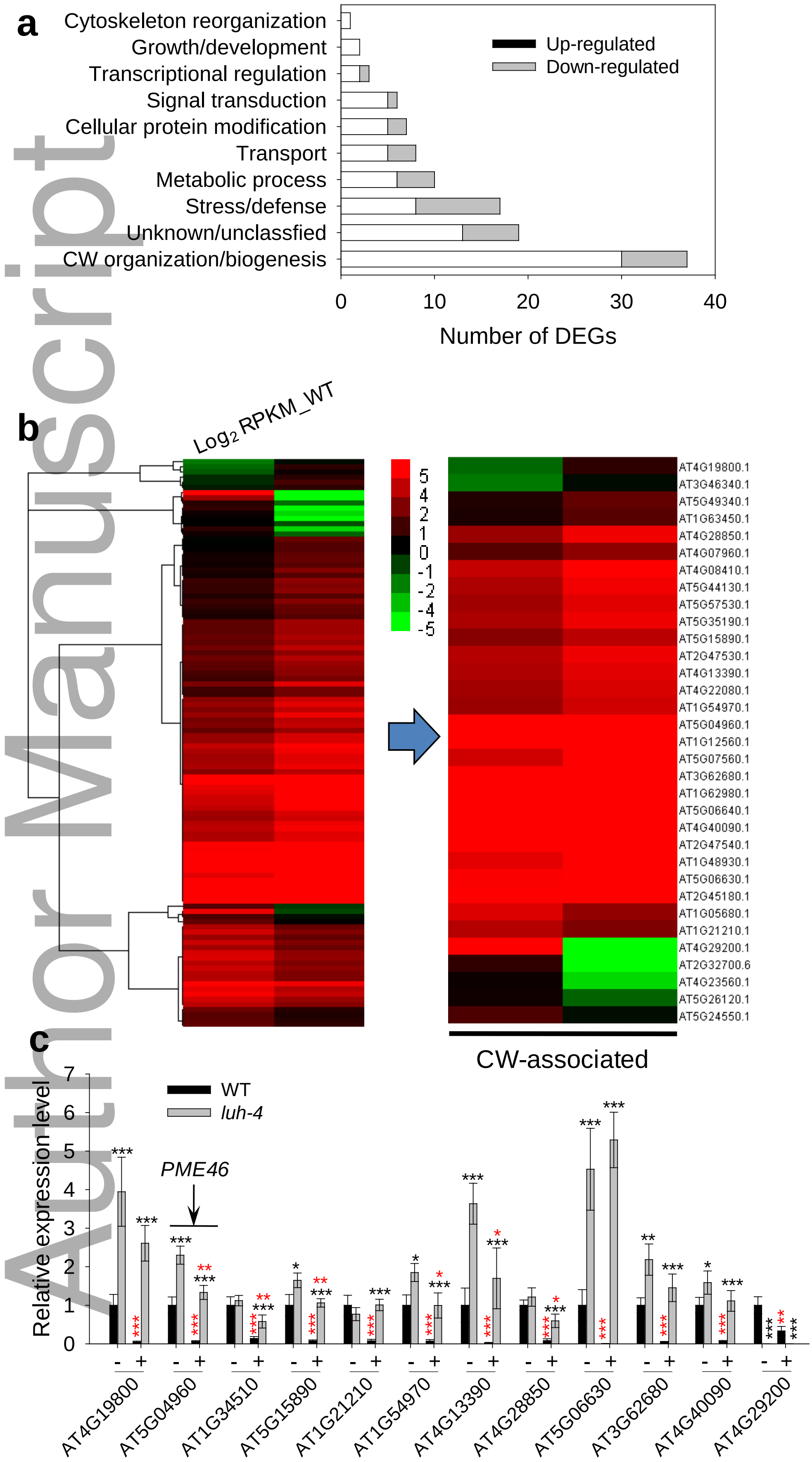
seedlings and mutants were grown into the hydroponic solution containing 0, 20 and 25 μM $AlCl_3$ at pH 4.2 for 7 days. Means represent values ±SD (n = 3). (b) Hematoxylin staining of Al distribution in root tips. (c) Al content in roots of WT seedlings and mutants. (d) Fractional analysis of cellular Al distribution in cell walls and symplast of Al-subjected roots of WT seedlings and mutants. Values in (c, d) represent means ± SD (n = 4). (e) qRT-PCR analysis of the expression of *PME46* in roots of WT seedlings and *slk2-1* mutant in response to Al stress. Six-day old WT seedlings and *slk2-1* mutant were treated without or with 25 μM $AlCl_3$ for 6 h. Values represent means ± SD (n = 3). *, ** and *** in (a, c, d, and e) indicate a significant difference between WT and mutant at $p < 0.05$, $p < 0.01$, and $p < 0.001$ (*t*-test), respectively.

Figure 7. Model of Al stress responses. It is proposed that *PME46* is a major inhibitor of PME activity and the expression of *PME46* is limited by the LUH/SLK2 complex. Under Al stress, *PME46* is mediated via two independent pathways: (1) unknown stress responsive factors and (2) LUH/SLK2 complex. Under Al stress: (i) in wild type plants, *PME46* expression is reduced leading to increased activity of PME (possibly through increased expression of other *PMEs*) and thus enhanced demethylation of pectin. Removal of methyl groups increases the negative charge of pectin and allows root cell walls to bind more Al. Al exposure also reduces expression of *LUH* leading to an elevated expression of *PME46*, while the elevation is insufficient to complement the suppression by Al via pathway (1); (ii) in *luh* and *slk2* (*luh*/*slk*) mutants, the mutation of LUH and SLK2 releases the expression of *PME46* from repression leading to lower PME activity, and thus less Al binding to root cell walls. However, it is presently not clear how elevated Al levels mediate *PME46* repression in pathway (1), but may involve the activity of yet to be identified stress response factors.


a
luh-4 (+1435)
luh-3 (+4387)
LUH
5'
3'
b
WT
luh-4
luh-3
UBQ1
LUH
c
Relative root growth (% of non-treated plants)
120
100
80
60
40
20
0
**
**
**
*
**
*
**
**
0
0.75
1
1.25
1.5
Al concentration (mM)
WT
luh-4
luh-3
d
WT
luh-4
luh-3
[AlCl$_3$] (μM)
0
25
e
Relative root growth (% of non-treated plants)
120
100
80
60
40
20
0
*
*
*
*
**
**
***
***
0
10
20
25
30
40
Al concentration (μM)
WT
luh-4
luh-3
f
Relative root growth (% of non-treated plants)
140
120
100
80
60
40
20
0
5.8
5.0
4.2
Solution pH
WT
luh-4
luh-3
g
Relative root growth (% of non-treated plants)
50
40
30
20
10
0
**
**
Al
Cu
Cd
La
WT
luh-4
luh-3


a
WT
luh-4
luh-3
b
Al content
[ng (5-mm root tips)$^{-1}$]
20
15
10
5
0
**
**
WT
luh-4
luh-3
c
Al content [µg g^{-1} FW]
60
50
40
30
20
10
0
**
**
WT
luh-4
luh-3
d
Cell-wall
Symplast
Al content [µg g^{-1} FW]
50
40
30
20
10
0
*
**
WT
luh-4
luh-3
e
Al binding capacity
[µg mg^{-1} DW]
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
**
*
WT
luh-4
luh-3


a
WT
luh-4
luh-3
WT
luh-4
luh-3
JIM5
JIM7
-Al
+Al
b
100
80
60
40
20
0
Fluorescence Intensity
-Al
+Al
*
***
*
**
**
*
**
WT
luh-4
luh-3
c
70
60
50
40
30
20
10
0
Fluorescence Intensity
-Al
+Al
**
***
**
**
**
***
*
WT
luh-4
luh-3
d
50
40
30
20
10
0
Pectin content
(μg GaE mg^{-1} DCW)
-Al
+Al
***
**
**
*
WT
luh-4
luh-3
e
60
50
40
30
20
10
0
Degree of methylation (%)
-Al
+Al
*
***
***
***
***
WT
luh-4
luh-3
f
40
30
20
10
0
Unmethylated pectin content
(μg GaE mg^{-1} DCW)
-Al
+Al
*
***
***
***
***
WT
luh-4
luh-3


a
Cytoskeleton reorganization
Growth/development
Transcriptional regulation
Signal transduction
Cellular protein modification
Transport
Metabolic process
Stress/defense
Unknown/unclassfied
CW organization/biogenesis
Up-regulated
Down-regulated
0
10
20
30
40
Number of DEGs
b
Log₂ RPKM_WT
5
4
2
1
0
-1
-2
-4
-5
AT4G19800.1
AT3G46340.1
AT5G49340.1
AT1G63450.1
AT4G28850.1
AT4G07960.1
AT4G08410.1
AT5G44130.1
AT5G57530.1
AT5G35190.1
AT5G15890.1
AT2G47530.1
AT4G13390.1
AT4G22080.1
AT1G54970.1
AT5G04960.1
AT1G12560.1
AT5G07560.1
AT3G62680.1
AT1G62980.1
AT5G06640.1
AT4G40090.1
AT2G47540.1
AT1G48930.1
AT5G06630.1
AT2G45180.1
AT1G05680.1
AT1G21210.1
AT4G29200.1
AT2G32700.6
AT4G23560.1
AT5G26120.1
AT5G24550.1
CW-associated
c
WT
luh-4
PME46
Relative expression level
0
1
2
3
4
5
6
7
- +
AT4G19800
AT5G04960
AT1G34510
AT5G15890
AT1G21210
AT1G54970
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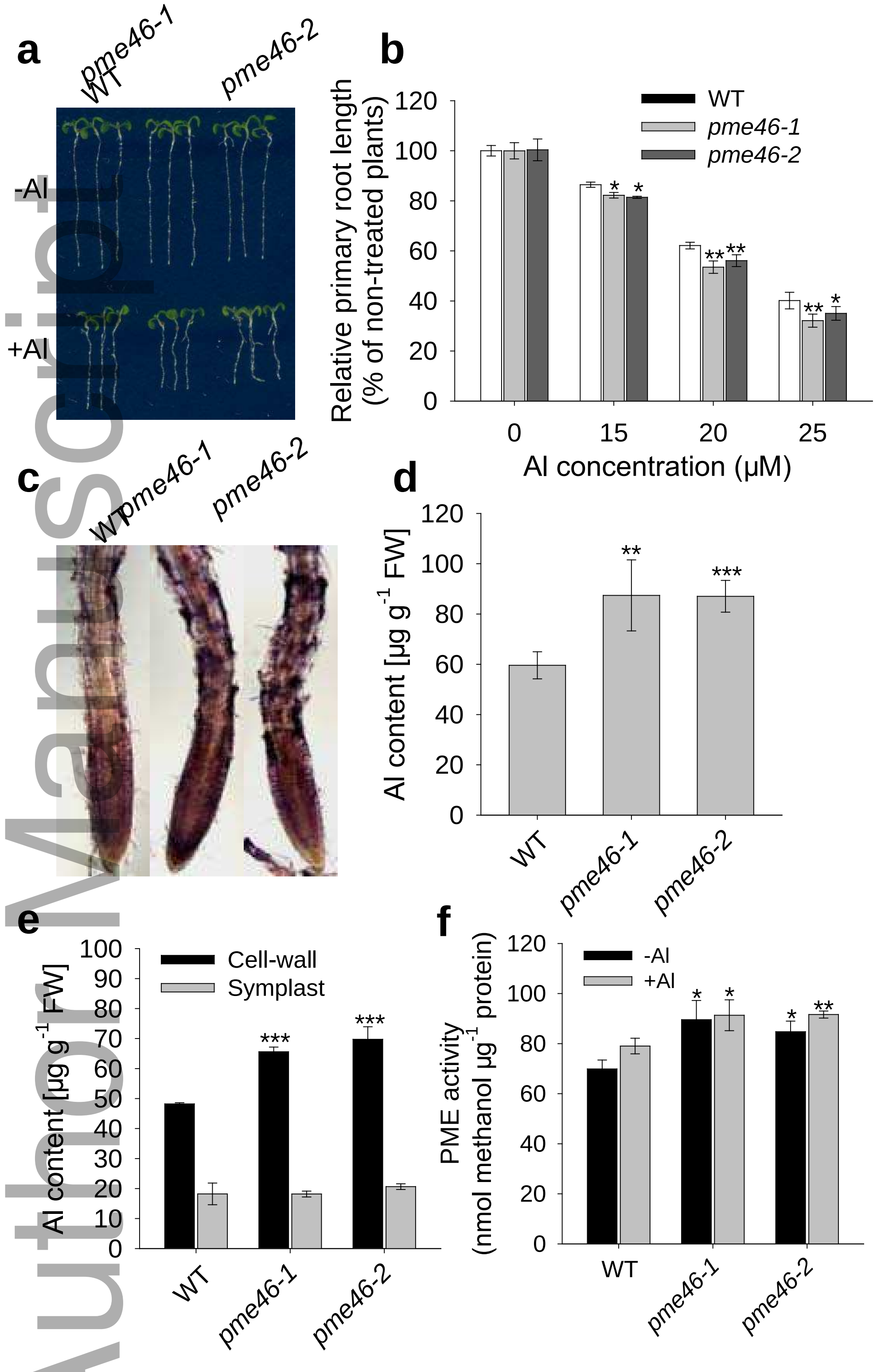
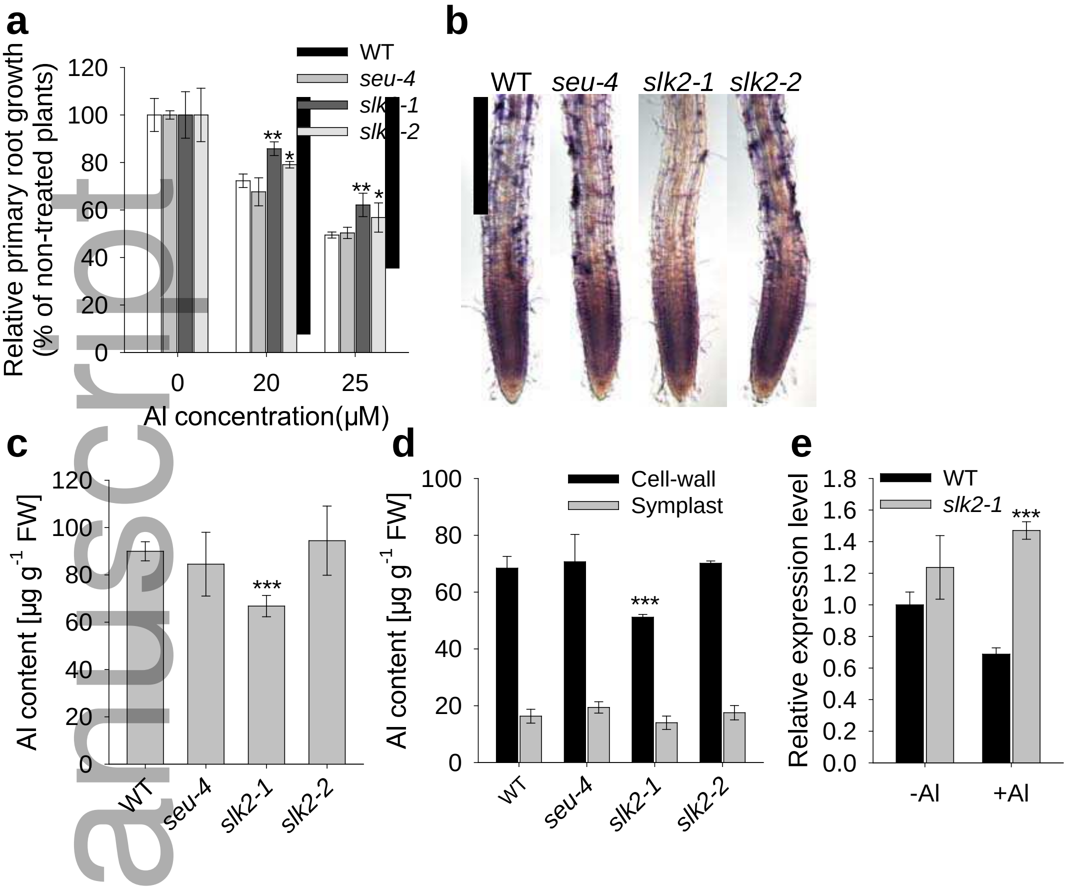
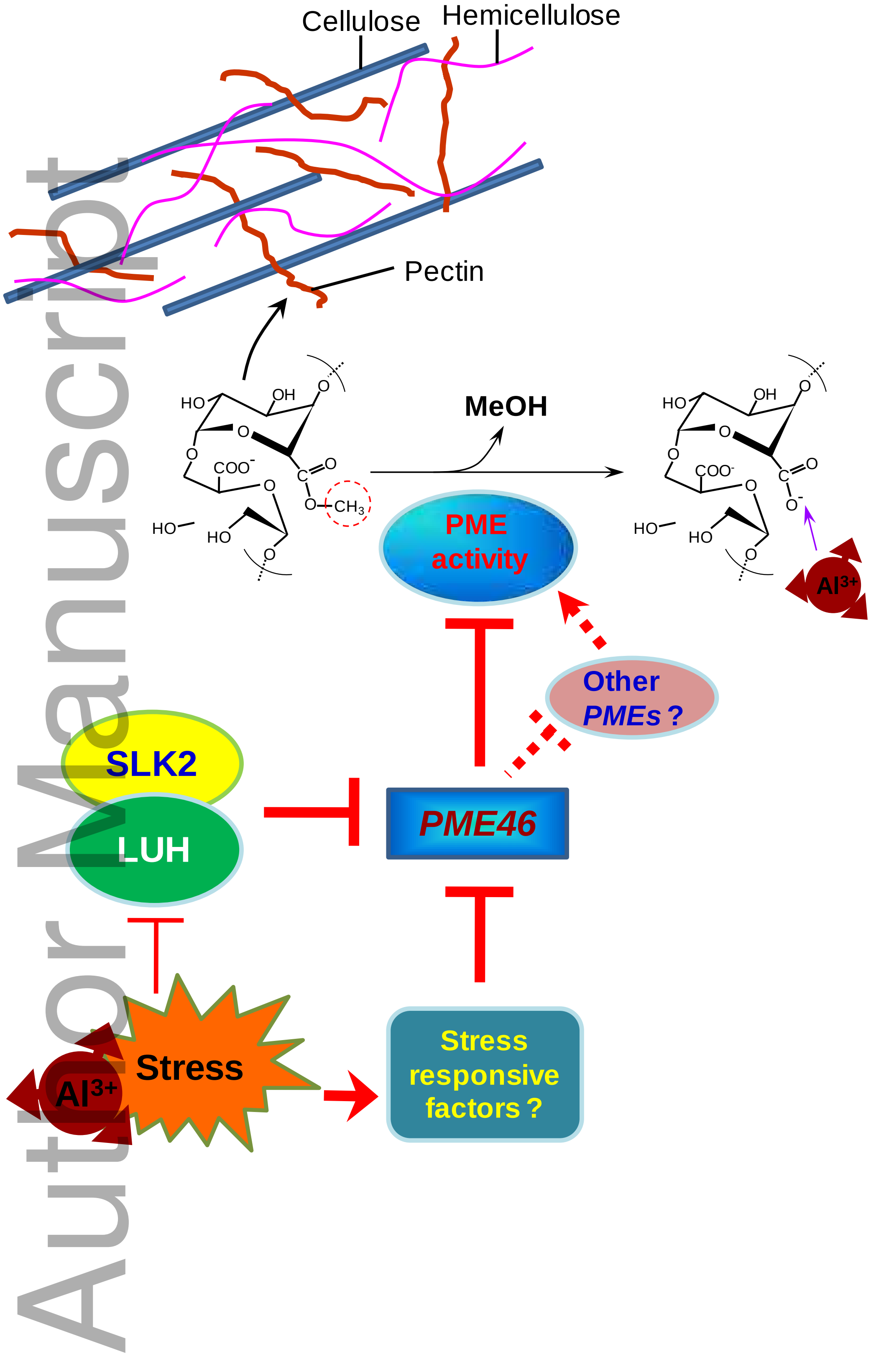
AT3G62680
AT4G40090
AT4G29200


a
WT
pme46-1
pme46-2
-Al
+Al
b
Relative primary root length (% of non-treated plants)
120
100
80
60
40
20
0
WT
pme46-1
pme46-2
*
*
**
**
**
*
0
15
20
25
Al concentration (μM)
c
WT
pme46-1
pme46-2
d
Al content [μg g-1 FW]
120
100
80
60
40
20
0
**
***
WT
pme46-1
pme46-2
e
Al content [μg g-1 FW]
100
90
80
70
60
50
40
30
20
10
0
Cell-wall
Symplast
***
***
WT
pme46-1
pme46-2
f
PME activity (nmol methanol μg-1 protein)
120
100
80
60
40
20
0
-Al
+Al
*
*
*
**
WT
pme46-1
pme46-2


a
WT
seu-4
slk -1
slk -2
Relative primary root growth
(% of non-treated plants)
120
100
80
60
40
20
0
**
*
**
*
0
20
25
Al concentration(μM)
b
WT
seu-4
slk2-1
slk2-2
c
Al content [μg g-1 FW]
120
100
80
60
40
20
0
***
WT
seu-4
slk2-1
slk2-2
d
Cell-wall
Symplast
Al content [μg g-1 FW]
100
80
60
40
20
0
***
WT
seu-4
slk2-1
slk2-2
e
WT
slk2-1
Relative expression level
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
-Al
+Al


Cellulose
Hemicellulose
Pectin
HO
OH
O
COO-
HO
HO
C=O
O-CH3
MeOH
PME activity
Other PMEs ?
SLK2
LUH
PME46
Stress
Al3+
Stress responsive factors ?
O-