



Changes in the Distribution of Pectin in Root Border Cells Under Aluminum Stress

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Root border cells (RBCs) surround the root apices in most plant species and are involved in the production of root exudates. We tested the relationship between pectin content in root tips and aluminum (Al) tolerance by comparing these parameters in wild-type (WT) and sensitive-to-Al-rhizotoxicity (*star1*) mutant rice plants. Staining for demethylesterified pectin decreased after Al treatment in the WT. A high level of pectin was observed in RBCs of the root tips. The level of total pectin was increased by about 50% compared with the control. In the Al-sensitive *star1* mutant, Al treatment decreased root elongation and pectin content, especially in RBCs. In addition, almost no Al accumulation was observed in the control, whereas more Al was accumulated in the RBCs of STAR1 roots. These results show that the amount of pectin influences Al tolerance; that Al accumulation in rice roots is reduced by the distribution of pectin in root-tip RBCs; and that these reactions occur in the field around the RBCs, including the surrounding mucilage. Al accumulation in rice roots is reduced by the distribution of pectin in root tips, and pectin in the root cell walls contributes to the acquisition of Al tolerance in rice by regulating its distribution. The release of Al-binding mucilage by RBCs could play a role in protecting root tips from Al-induced cellular damage.

Keywords: aluminium, root border cells, pectin, mucilage, rice (*Oryza sativa* L.)

INTRODUCTION

Aluminum (Al) is the most abundant metal in the Earth's crust. Under acidic conditions, Al is solubilized to its ionic form, which is toxic to plants (Foy, 1988). Al rapidly inhibits root elongation and, subsequently, the uptake of water and nutrients, resulting in significant reductions in crop production in acidic soils, which comprise 30% to 40% of the world's arable soils (Von Uexküll and Mutert, 1995). Al compounds are affected by the pH of coexisting elements and soil. Under normal environmental conditions, Al assumes the form of $\text{Al}(\text{OH})_4^-$; with decreasing pH, its morphology changes to $\text{Al}(\text{OH})_3$, $\text{Al}(\text{OH})_2$, and $\text{Al}(\text{OH})_2^+$ (Duan and Gregory, 2003). In an acidic soil with pH <5, it elutes in the form of the water-soluble ion Al^{3+} , which is believed to most strongly inhibit the growth of plants when absorbed (Famoso et al., 2010). Growth inhibition by Al has been reported in various species, including rice, wheat, corn, rye, beet, mushroom, tomato, and *Arabidopsis thaliana* (Searcy and Mulcahy, 1990; Li et al., 2000; Famoso et al., 2010; Parra-Almuna et al., 2018;

Abbreviations: RBC, root border cell; STAR, sensitive to Al rhizotoxicity; WT, wild type.

Xu et al., 2018). The toxicity mechanisms of Al are complex, and the exact mechanism by which Al initially causes the inhibition of root elongation is not fully understood (Kochian et al., 2005). However, most Al-related events clearly result from the binding of Al to extracellular and intracellular substances because of the high affinity of Al for oxygen donor compounds. Most Al inhibiting root elongation are localized to cell walls in the epidermis and outer cortex (Jones et al., 2006).

Some studies of the localization of Al in the cell wall have involved the examination of its relationship to pectin (Chang et al., 1999) and hemicellulose (Yang et al., 2011; Zhu et al., 2017a; Zhu et al., 2017b; Xu et al., 2018) contents. Meanwhile, some plant species have developed mechanisms to cope with internal and external Al toxicity (Ma et al., 2001; Ryan et al., 2001; Rengel, 2004; Kochian et al., 2005). Some plants are Al tolerant, and several Al resistance mechanisms have been proposed in previous studies. For example, buckwheat has been shown to transport absorbed Al to the aerial part of the plant, which then accumulates in vacuoles in a state chelated to oxalic acid (Wang et al., 2015). The most documented mechanism of Al resistance is the secretion of organic acid anions from plant roots (Li et al., 2000; Ma, 2000; Ma et al., 2001; Ryan et al., 2001; Kochian et al., 2005; Hoekenga et al., 2006; Kopitke et al., 2017). Root border cells (RBCs) and mucilage have also been suggested to increase Al tolerance in plants (Cai et al., 2011; Yang et al., 2016). RBCs are living cells that detach from the root cap and are considered to be involved in stress responses to the soil and produce hydrophilic polysaccharides in the soil. Polysaccharides secreted in the soil are hydrated and become mucilage substances (Hawes and Lin, 1990; Yu et al., 2009). RBCs are suggested to have different gene expression and metabolism pattern from cells in root tips (Watson et al., 2015). RBCs and mucilages have been shown to protect root tips from chemical and biological stresses, such as iron toxicity (Xing et al., 2008) and fungal infection (Hawes et al., 2000; Gunawardena and Hawes, 2002). Previous reports showed that the presence of RBCs and mucilages decreases Al accumulation and increases Al tolerance (Barcelo and Poschenrieder, 2002; Curlango-Rivera et al., 2016; Hawes et al., 2016). Al-resistant plants tend to have less Al accumulation than do Al-sensitive plants (Ma et al., 2005). In addition, the cell wall has been shown to readily adsorb and bind to Al (Van et al., 1994). Rice is one of the world's major crops, and it has shown relatively high Al tolerance (Famoso et al., 2010). However, the mechanism responsible for Al tolerance in rice is not well understood. Rye has been suggested to have Al resistance equivalent to that of rice, with organic acid secretion from the root ends (Li et al., 2000). Increased Al concentrations also increase citric acid secretion in rice, with no significant difference in this secretion between Al-resistant varieties (Koshihikari) and Al-sensitive varieties (Katalath) (Ma et al., 2002). Moreover, no significant decrease in Al tolerance has been observed in mutants with little organic acid secretion (Yokosho et al., 2016). These findings suggest that the secretion of organic acids is not strongly involved in the Al resistance of rice. In recent years, gene expression studies have been undertaken to investigate the mechanism of Al tolerance in rice, and the transcription factor ADP-ribosyltransferase 1 (ART1), whose expression is increased by Al stress, has been identified. Furthermore, the sensitive to Al rhizotoxicity 1 (STAR1) gene encoding the ABC transporter

has been shown to be among several genes whose expression is controlled by ART1 (Huang et al., 2009). These genes are thought to contribute to rice Al resistance (Delhaize et al., 2012), as ART1 and the *star1* mutants are strongly susceptible to growth inhibition by Al absorption (Delhaize et al., 2012). The *star1* mutant has a DNA region that is thought to encode the nucleotide-binding domain. It is expressed throughout the roots, excluding the mature epidermis, suggesting localization to vesicles in cells. STAR1 has been suggested to form a complex with STAR2, which has a transmembrane domain and specifically transports UDP glucose. UDP glucose transported outside of cells from vesicles is used for the modification of cell wall polysaccharides, and Al stress is thought to be reduced by the modification of binding between Al and these polysaccharides (Huang et al., 2009). In a study comparing changes in gene expression in response to Al treatment (Tsutsui et al., 2012), elevated expression of galacturonosyltransferase, a gene involved in the biosynthesis of pectin 1 to 2 cm from the root tips, was observed in wild-type (WT) but not in STAR1 plants.

In this study, we examined the relationship between pectin distribution in root tips and plant Al tolerance using hydroponics. The Al concentration we used was higher than the acid soil, but consistent with previous Al toxicity researches in rice. Our results show that the pectin distribution influences Al tolerance, that Al accumulation in rice roots is reduced by the distribution of pectin in root tips, and that pectin in the root cell walls contributes to the acquisition of Al tolerance in rice by regulating its distribution.

MATERIALS AND METHODS

Plant and Growth Conditions

Nipponbare, Koshihikari, and *star1* mutant of rice (*Oryza sativa*) were used in this study (Huang et al., 2009). After imbibition in ion exchanged water for 1 or 3 days at 30°C, rice seedlings were grown on floating net on 1.0 or 0.5 mM CaCl₂, pH 4.5 for 3 days. Grown seedlings were exposed to Al with 1.0 or 0.5 mM CaCl₂ and 0, 50, or 100 μM AlCl₃, pH 4.5 water culture media for 1 day in 15-ml centrifuge tube. Free Al activities were evaluated by using GEOCHEM-EZ software (Yoshida et al., 1972; Shaff et al., 2010) and values are between 76.57% and 78.28%. They were grown at 30°C under continuous light at 250 μmol m⁻² s⁻¹.

Measurement of Root Elongation

Root length was measured before and after Al treatment with a ruler to calculate root elongation during Al treatment. Relative root elongation, RRE (%) = (root growth in each Al condition)/(root growth in control) × 100 was calculated to compare root elongation and Al tolerance between different lines (Furukawa et al., 2007).

Saponification of Pectin

To remove the methyl groups from pectin and change methylesterified pectin to demethylesterified pectin, methylesterified pectin in roots was saponified with 0.1 N NaOH in 50-ml centrifuge tube for 1 min. After saponification, roots

were washed with ion exchanged water, and then all pectin can stain by ruthenium red (Iwai et al., 1999).

Staining Demethylated Pectin With Ruthenium Red

To detect the demethylesterified pectin in roots, sample roots were stained with 0.01% (w/w) ruthenium red in 50-ml centrifuge tube for 5 min. After staining, roots were washed with ion exchanged water (Iwai et al., 1999).

Staining Al With Eriochrome Cyanine R

Al in roots was stained with 0.1% (w/w) eriochrome cyanine R in 50-ml centrifuge tube for 20 min. After staining, roots were washed with ion exchanged water (Jones and Thurman, 1957).

Collecting Cell Wall

Root tips (0–1 mm) from three seedlings were cut with a razor and collected in a 2.0-ml tube as a cell wall sample. Samples were frozen in liquid nitrogen and crushed with pestles.

A series of processes was repeated twice, adding methanol/chloroform mixture (1 ml, 1:1, v/v), centrifuging at 15,000 rpm for 5 min and removing supernatant from samples. After the last supernatant removal, samples were air dried (Sumiyoshi et al., 2013).

Collecting Water Culture Media

After Al treatment, water culture media was frozen at -80°C and lyophilized (FDU-2200; Tokyo Rikakikai Co, Ltd) at 3.3 Pa for 8 days (Iwai et al., 2003).

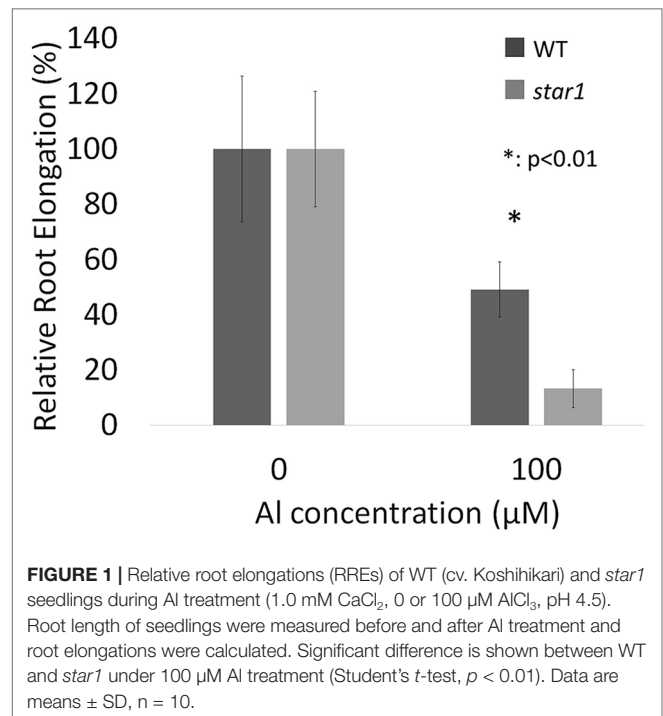
Determination of Uronic Acid

Uronic acid was determined by the method of Blumenkrantz and Gustav (1973). One milliliter of ion exchanged water was added into each sample, and 1 ml of iced concentrated sulfuric acid (0.025M borax) was mixed into 200 μl of each sample. After heating in 100°C water for 10 min and cooling in ice, 40 μl of carbazole solution (carbazole 125 mg/ethanol 100 ml) was mixed into each sample. Samples were heated in 100°C water for 15 min and cooled in ice to measure the absorbances at 530 nm (GENESIS 10S UV-VIS; Thermo Scientific).

RESULTS

Distribution of Pectin in *star1* Mutant Roots After Al Treatment

Seedlings grown for 3 to 4 days with culture media containing 0.5 and 1.0 mM CaCl_2 (pH 4.5) were treated for 1 day with culture media containing 0, 50, and 100 μM AlCl_3 . The elongation of roots during Al treatment was calculated using pre- and post-treatment root length measurements. Relative elongation of 100 μM Al-treated roots was reduced by about 70% in the *star1* mutant compared with the WT, and the root elongation of the *star1* mutant was significantly low (*t*-test, $p < 0.01$; Figure 1). After treatment, the control WT roots were stained with ruthenium red, indicating the presence of demethylesterified pectin, 0.2 mm from the root tips. Staining revealed the presence of demethylesterified pectin in the roots of the WT and *star1*



mutant after Al treatment. RBCs of WT roots treated with 0- μM Al also showed staining. However, pectin staining distribution of WT RBCs and roots treated with 50- and 100- μM Al narrowed (Figure 2A). All Al treatments narrowed demethylesterified pectin staining distribution of STAR1 RBCs and roots (Figure 3A). Whole roots showed staining for saponified pectin (total pectin); in roots treated with high Al concentrations, an increase in staining was observed on the root side of the root cap (Figure 2B). In comparison with Al-treated WT (Koshihikari) roots, Al-treated roots STAR1 showed suppression of the increased staining in the root-side section beyond 1 mm from the tip (Figure 3B). A significant difference was not observed in the alteration in distribution of demethylesterified pectin (Supplemental Figure 1A) or all pectin (Supplemental Figure 1B) in roots by Al treatment between Nipponbare and Koshihikari.

Distribution of Al in *star1* Mutant Roots After Al Treatment

After Al treatment, the WT (Koshihikari) and STAR1 roots were stained with eriochrome cyanine R. Little staining was observed in the WT RBCs. In STAR1, strong staining by eriochrome cyanine R was observed in many regions of the root and RBCs (Figure 4). In particular, staining was stronger in RBCs than in other root regions.

Quantification of Pectin in Cell Walls and Culture Media

The amounts of total pectin contained in the cell walls 1 mm from the root tips in control and Al-treated plants were quantified by the carbazole-sulfuric acid method. Pectin contents were

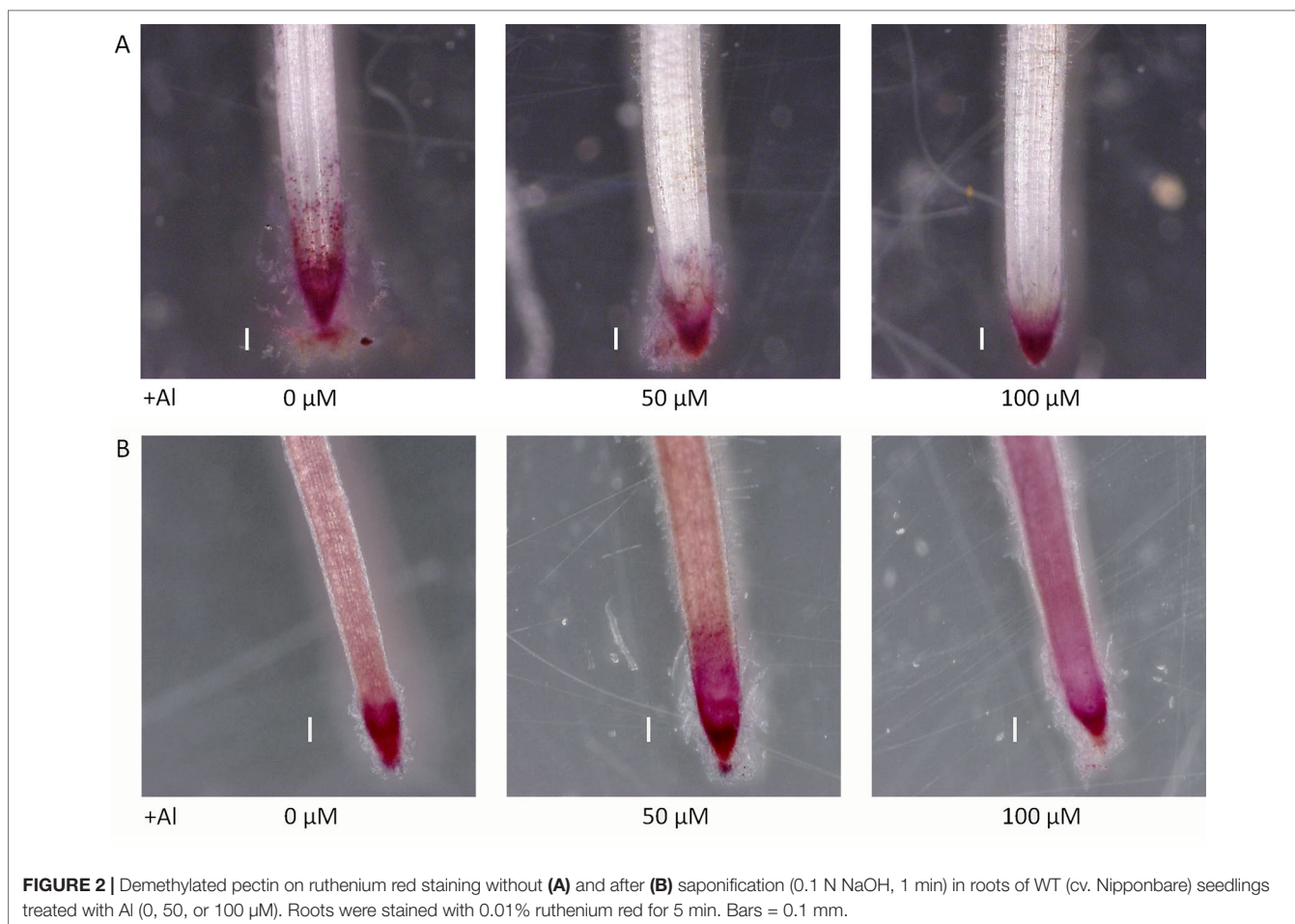


FIGURE 2 | Demethylated pectin on ruthenium red staining without **(A)** and after **(B)** saponification (0.1 N NaOH, 1 min) in roots of WT (cv. Nipponbare) seedlings treated with Al (0, 50, or 100 μM). Roots were stained with 0.01% ruthenium red for 5 min. Bars = 0.1 mm.

increased by about 50% in Al-treated plants compared with the control, suggesting that Al treatment increased the pectin content in root ends (**Figure 5**).

The pectin content of culture media did not differ significantly according to Al treatment or plant strain (**Figure 6**).

DISCUSSION

The root tip is the primary site of Al toxicity in higher plants (Ryan et al., 1993), and its encasing mucilaginous capsule has been implicated as a protective source of materials that prevent the uptake of Al into root meristems (Horst et al., 1982). To our knowledge, the physiological mechanism by which Al damages cells is not known (Kochian, 1995). The root cell wall has been suggested to be a site of Al toxicity and Al exclusion (Horst et al., 2010). Up to 90% of the Al absorbed by roots can be localized to the apoplast (Kochian, 1995). The primary site of Al^{3+} binding is probably the pectin matrix, which is composed largely of homopolymers of galacturonic acid (Mohnen, 2008; Horst et al., 2010). During plant development, cell wall pectin biosynthesis and assembly occur, and pectins secreted to the apoplast are highly esterified and later de-esterified by the activity of pectin methyltransferase, inducing pectin–Ca crosslinking, which has an important role in the cell wall. Al^{3+} is known to bind far more

strongly to pectin than to Ca^{2+} , whose binding to the cell wall is required for proper cell wall functioning (Franco et al., 2002). Al treatment has been shown to increase the pectin content in the roots of pumpkin (Van et al., 1994), suggesting that pectin is involved in the Al stress response. In addition, the pectin distribution in response to Al and its possible impact on the Al resistance of RBCs had not been examined. In this study, an Al-sensitive mutant was used to test the hypothesis that RBCs and the pectin that they produce are involved in the detection and avoidance of Al toxicity. The Al concentration we used was higher than the exchangeable Al concentration in acid soil. However, some previous Al toxicity researches in rice employed around 100 μM Al^{3+} (Huang et al., 2009; Famoso et al., 2010; Cai et al., 2011; Maejima et al., 2014). Therefore, the range of Al concentration we used in this study is consistent with those.

The demethylesterified pectin distribution of roots, and especially of RBCs, narrowed with Al treatment in a dose-dependent manner (**Figure 2A**). Initially, we considered the hypothesis that the Al tolerance of rice could be increased by decreasing the demethylesterified pectin content. However, the type II cell walls of monocotyledonous plants, including rice, contain little pectin (Yokoyama and Nishitani, 2004); dicotyledonous plants with type I cell walls are pectin rich (Yang et al., 2011). Thus, proving that a high demethylesterified pectin

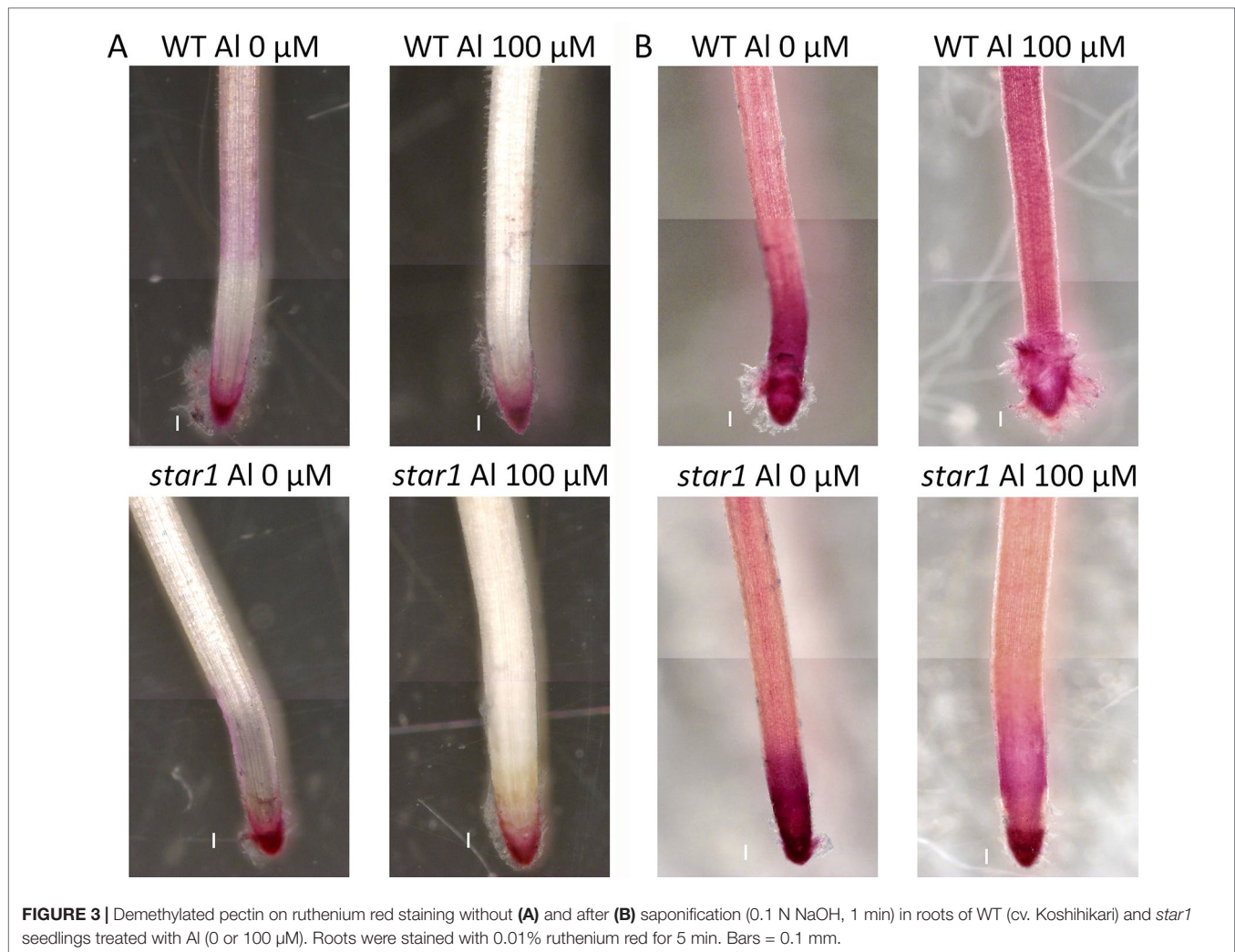


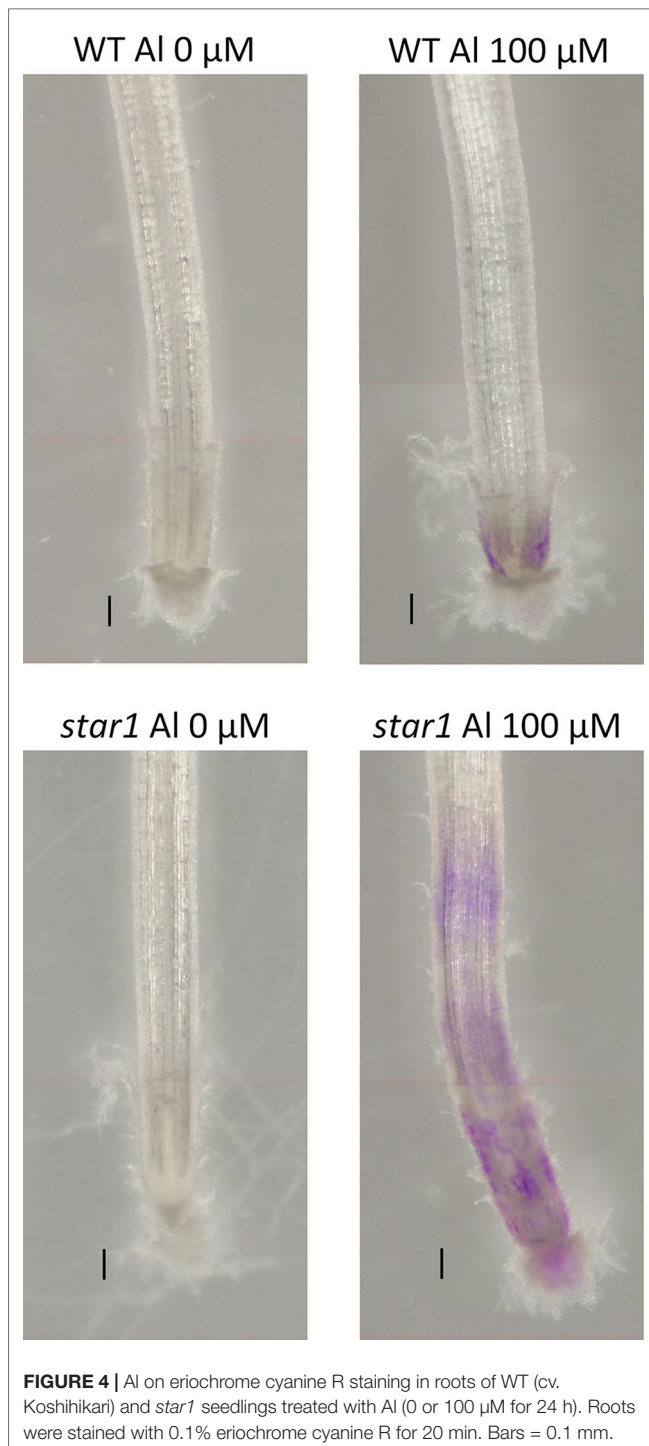
FIGURE 3 | Demethylated pectin on ruthenium red staining without (A) and after (B) saponification (0.1 N NaOH, 1 min) in roots of WT (cv. Koshihikari) and *star1* seedlings treated with Al (0 or 100 μM). Roots were stained with 0.01% ruthenium red for 5 min. Bars = 0.1 mm.

content alone, which could increase the Al tolerance of rice, was difficult.

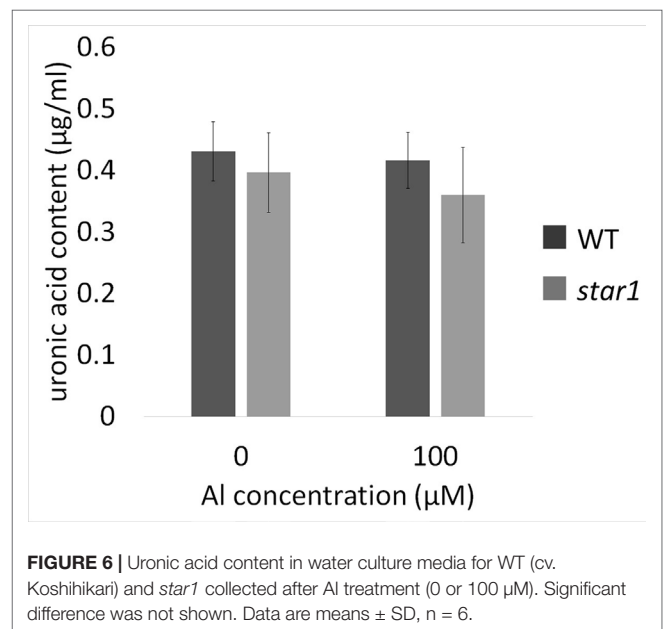
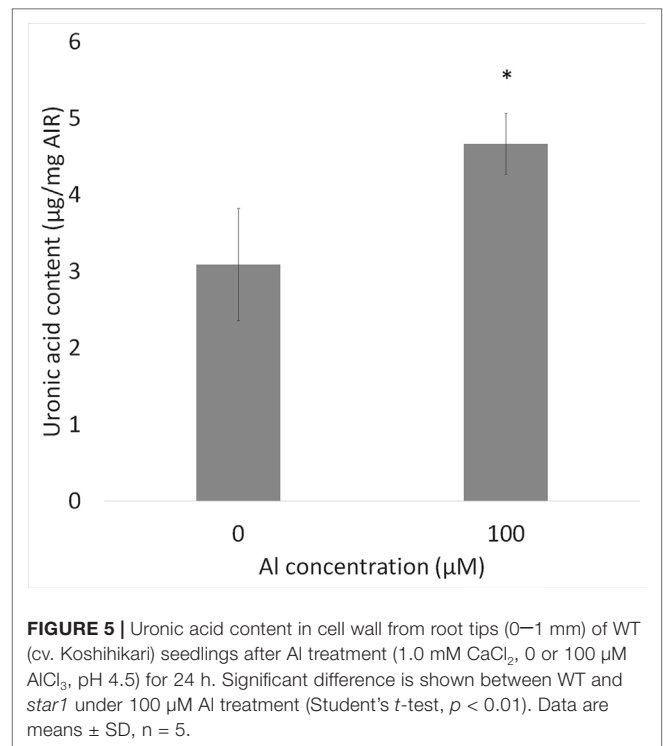
Therefore, we focused on the distribution and function, instead of the amount of demethylesterified pectin. Our results showed that RBC pectin distribution was related closely to Al concentrations in the culture media (Figure 2A). Therefore, active RBC release demethylesterified pectin in root tips, and subsequently, separated RBCs can absorb Al in culture media. This RBC behavior may block the accumulation of Al in root tips. However, the pectin content of culture media did not differ significantly according to the presence or absence of Al treatment in STAR1 or WT samples (Figure 6). Thus, demethylesterified pectin produced by RBCs and secreted in the culture medium does not contribute to the reduction of Al toxicity.

Hemicellulose is among the polysaccharide constituents of the cell wall. This polysaccharide is fractionated from the cell wall using potassium hydroxide after the fractionation of pectin (Yang et al., 2011; Zhu et al., 2017a; Zhu et al., 2017b). Hemicellulose interacts with cellulose fiber by hydrogen bonding and is covalently bonded to hemicellulose on neighboring cellulose fibers, forming a chain of pectic polymers (Scheller and Ulvskov,

2010). Hemicellulose polysaccharides are classified as xyloglucan, xylan, β -(1-3), (1-4)-glucan, callose, and arabinogalactan, and they play an important role in cell elongation *via* cell wall loosening (Scheller and Ulvskov, 2010). The cell walls of monocotyledonous plants, including rice, contain 30% to 70% hemicellulose in their primary cell walls (Scheller and Ulvskov, 2010). As hemicellulose has been found to adsorb Al preferentially to pectin (Yang et al., 2011; Zhu et al., 2017a; Zhu et al., 2017b), we compared the distributions of demethylesterified pectin, total pectin, and adsorbed Al. On eriochrome cyanine R staining, the region that adsorbs Al appeared not to overlap with the region that contained demethylesterified pectin or total pectin. In WT plants containing high levels of demethylesterified pectin, Al-treated roots showed almost no accumulation of Al (Figure 4). On the other hand, Al accumulation was found in a wide range of root caps and RBCs in the *star1* mutant (Figure 4). Although the distribution of demethylesterified pectin in roots was detected in the WT (Figures 2A and 3A), these roots showed little Al accumulation (Figure 4). These results suggest that demethylesterified pectin in WT RBCs is crosslinked mainly with Ca and linked slightly to Al³⁺. Al³⁺ may stick to Ca-bonded pectin, which acts as a cation exchange resin



because Ca bonding of pectin is irreversible and Al^{3+} binding to cell-wall pectin through replacement of Ca^{2+} is impossible. In addition, alkali-soluble pectin contained in RBCs appears to adsorb Al in the early stage of growth inhibition in pea (Yang et al., 2016). Pectin responsible for cell wall adhesion between cells is actively demethylesterified by pectin methylesterase (PME) and degraded by polygalacturonase (PG), which is thought to cause RBCs to fall out of the root cap (Stephenson and Hawes, 1994; Wen



et al., 1999). RBCs, including those with Al bound to Ca-bonded pectin, were separated and released to the culture medium. On the other hand, the accumulation of Al in hemicellulose has been suggested to increase in the AtSTAR1 function-deficient mutant of *A. thaliana* (Xu et al., 2018). Our results suggest that RBCs in the *star1* mutant have less pectin. Therefore, the hemicellulose, rather than pectin, in the cell walls of STAR1 accumulates Al. Our results are consistent with previous reports that Al-sensitive

mutants and cultivars accumulate more Al than do controls (Ma et al., 2002; Ma et al., 2005) (Figure 4). Rice PME expression increases in Al-treated roots; Al accumulation in roots increases in PME-overexpressing rice, and Al tolerance decreases (Yang et al., 2013). Pectin was actively demethylesterified by overexpression of PME, became susceptible to decomposition by PG, and its root content was reduced while RBCs elimination was promoted, increasing Al resistance through the pectin in RBCs. The function of this mechanism is considered to decrease with increasing Al accumulation (Yang et al., 2013). Our results suggest that Al accumulation in rice roots is reduced by the distribution of pectin in root tips, and that pectin in the root cell walls contributes to the acquisition of Al tolerance in rice by regulating its quantity and distribution. These pectin-related barriers to Al reduce Al toxicity. The release of Al-binding mucilage by RBCs could play a role in protecting root tips from Al-induced cellular damage.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in University of Tsukuba Repository, <https://tsukuba.repo.nii.ac.jp>.

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AUTHOR CONTRIBUTIONS

TN, AN, JF, and HI conceived the experiment(s), analyzed the result(s), and wrote the paper. JF and HI conducted the experiment(s). All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2019.01216/full#supplementary-material>

FIGURE S1 | Demethylated pectin on ruthenium red staining without (A) and after (B) saponification (0.1 N NaOH 1 min) in roots of WT (cv. Koshihikari) seedlings treated with Al (0, 100 μ M). Roots were stained with 0.01% ruthenium red for 5 min. Bars = 0.1 mm.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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