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Exogenous proline regulates pectin demethylation by rescuing pectin methylesterase functioning of cell wall from Cr(VI) toxicity in rice plants

Abid Ullah[1†], Yu-Juan Lin[1†], Peng Tian[1] and Xiao-Zhang Yu[1*]

Abstract

Background Plants are equipped with several sophisticated mechanisms to deal with heavy metals (HMs) toxicity. Cell walls, which are rich in pectin, are important in the sequestration and compartmentalization of HMs. Pectin demethylation is carried out by pectin methylesterase (PME), which is a crucial activity in cell walls for the adsorption of HMs. This study focused on the factors that contribute to chromium (Cr) adsorption in rice plants exposed to Cr(VI) treatments without proline (Pro) "Cr(VI)" and with Pro "Pro + Cr(VI)" application.

Results The results exhibited that when rice plants were treated with Cr(VI), their PME activity decreased, because Cr(VI) was bound to certain isoforms of PME and prevented the demethylation of pectin. The application of Pro increased PME activity by promoting the transcription of several PME-related genes. These genes were recognized on the basis of their similarity with PME genes in *Arabidopsis*. Gene expression variation factors (*GEVFs*) between the "Cr(VI)" and "Pro + Cr(VI)" treatments revealed that *OsPME7* and *OsPME9* have the highest positive *GEVF* values than other *OsPME* genes of rice. In addition, Pro application increased pectin content significantly in rice plants exposed to Cr(VI) stress. Proline application also leads to an increased concentration of Cr in rice roots compared with "Cr(VI)" treatments alone.

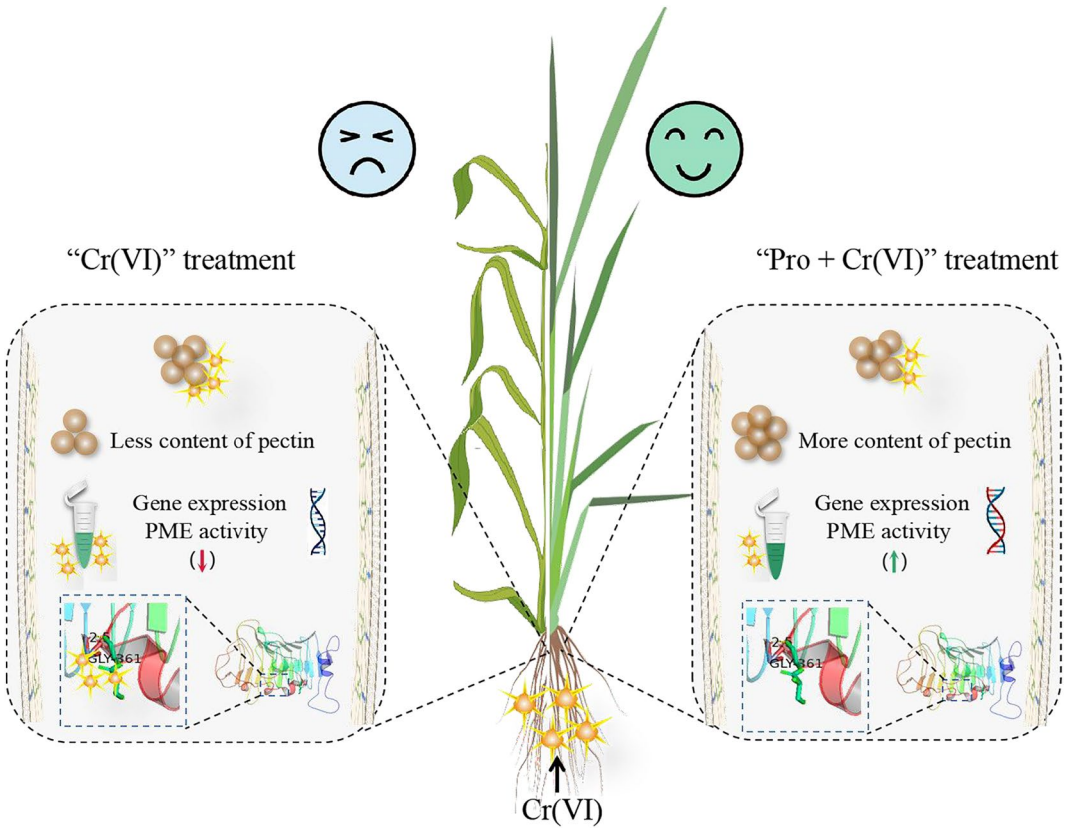
Conclusions These findings suggest that Pro increased Cr(VI) adsorption in cell walls of rice plants by enhancing the PME activity and pectin content when exposed to "Cr(VI)" treatments, mainly regulated by *OsPME7* and *OsPME9*.

Keywords Cell wall, Chromium, Rice, Proline, Pectin methylesterase

[†]Abid Ullah and Yu-Juan Lin have contributed equally to this work.

*Correspondence:
Xiao-Zhang Yu
xzyu@glut.edu.cn
Full list of author information is available at the end of the article

Graphical Abstract



Introduction

Heavy metals (HMs) are well-known environmental pollutants due to their toxic nature. The accumulation of HMs in plants leads to toxicity, resulting in damage to the photosynthetic apparatus, disruption of ionic balance, increased production of reactive oxygen species (ROS), inhibition of enzymatic activities, and impaired nutrient uptake, collectively contribute to a reduction in plant growth and development. However, plants are equipped with several sophisticated mechanisms to deal with the toxicity of HMs, including chelation, exclusion, immobilization, sequestration, and compartmentalization into cellular organelles [1]. Cell walls, which are rich in polysaccharides such as hemicellulose and pectin, are important in the sequestration and compartmentalization of HMs [2]. Pectin is a highly complex form of polysaccharide in terms of both structure and function; accounting for 35% of the primary walls of dicotyledons and non-graminaceous monocotyledons, 2–10% of the primary walls of grasses and other clumps, and up to 5% of the xylem tissues [3]. Pectin polysaccharide serves as the primary site for HM binding and accumulation because homogalacturonan (galacturonic acid) is present in the cell wall together with rhamnogalacturonan-I and rhamnogalacturonan-II [4]. In pectin structures, the methyl group ($-CH_3$) is typically bonded to the C-6 position of the galacturonic acid residues. Homogalacturonan is a methylesterified form of pectin that is found in cell walls [1]. Demethylation of pectin catalyzed by pectin methylesterase (PME) could increase the abundance of functional groups in pectin, namely, –COOH, –OH, and –SH occurring in the cell wall, and subsequently enhance the capacity of the cell wall for binding HMs [5]. It is established that the degree of pectin demethylesterification corresponds to the PME activity, the stronger the PME activity, the higher the level of pectin demethylesterification in plants [6]. Exposing plants to HMs can increase PME activity in cell walls up to a certain threshold, resulting in a reduction of methoxylated pectin levels and subsequently altering the degree of pectin methylesterification, which may impact the number of binding sites for HMs in cell walls [7].

Chromium (Cr) is one of the most hazardous metals, ranking in the top 20 toxic materials in the world. There are seven (0–VI) oxidation states of Cr, but its hexavalent form [Cr(VI)] is particularly stable and toxic species for all living organisms [8]. According to Murad et al. [9], 896 tonnes of Cr is exposed to the soil every year from different anthropogenic sources, particularly industrial processes. These industries include steel, minerals, chemicals manufacturing, leather tanning, metal plating, cement, electroplating, and textile dyeing [10]. Therefore, the concentration of Cr in the soil is increasing worldwide due to anthropogenic and/or natural sources [11]. Unfortunately, Cr is environmentally persistent and non-biodegradable, leading to its accumulation and widespread distribution in the environment over time [12]. Its higher concentration in the arable soil matrix suppresses seed germination, alters nutrient balance, represses photosynthesis and respiration, increases lipid peroxidation, and induces oxidative damage in crop plants [13]. Plants avoid Cr toxicity by several mechanisms, including the use of cell walls to intercept metal ions outside the cell, employ efflux transporters to remove Cr ions from cells, chelation and sequesteration of metal ions into subcellular compartments, and reduce its uptake and translocation [14]. Cr is absorbed by plant roots, which is largely retained in the root system, and a very small fraction is transferred to the shoots to minimize damage in the upper parts. In the roots, metal ions are mostly sequestered in cell walls by binding with different functional groups present there [2, 15].

Several strategies are being used to mitigate Cr toxicity in plants, specifically its accumulation in the shoot which can be penetrated in the food chain. Among these, proline (Pro) is frequently used to mitigate Cr toxicity in plants, because it acts as an osmolyte, an antioxidative molecule, a metal chelator, and a signaling molecule in plants [16]. Exogenous Pro has been used effectively to enhance HMs tolerance in several plants [13]. As reported previously, the Pro application enhanced resistance against Cd in olive plants by improving antioxidant enzyme activities, nutrient availability, photosynthetic activity, and other parameters of plant growth [17]. Similarly, Pro mitigated the toxicity of Se by improving antioxidant systems and plant growth parameters in bean seedlings [18]. From their results, we hypothesized that Pro may play a regulatory role in Cr(VI) stress alleviation through the demethylation of pectin catalyzed by PME. Thereby, we aimed to clarify the mechanism of exogenous Pro-induced modification of PME functioning in Cr(VI) tolerance by enhancing the demethylation of pectin in rice plants. To achieve this, we integrated the biochemical and molecular analysis (i.e., Cr accumulation, pectin content, PME activity, and expression of PME-associated genes) with molecular docking and scoring function estimation of the affinity potential to elucidate how and why Cr(VI) repress PME activity while exogenous Pro rescue it and enhance Cr(VI) adsorption in cell walls through enhancing the demethylation of pectin in rice seedlings.

Materials and methods

Plant cultivation and chemical preparation

The rice seedlings (*Oryza sativa* L. XZX 45) were produced following the method described by Zhang et al. [13]. Briefly, the seeds were soaked in double distilled water for 12 h and then placed in plastic pots filled with river sand under saturated soil conditions. These pots were then transferred to an incubator set on a temperature of 25 ± 0.5 °C, a relative humidity of 65 ± 2%, and constant light. The 8692 nutrition solution with slight changes was used for watering to keep the nutrient balance in the soil. The nutritional composition of the "8692 nutrient solution" is provided in Table S1 of supporting information. After 16 days of pre-growth, the seedlings were thoroughly cleansed with double distilled water and ionic removing buffer for the following treatments. (1) "Cr(VI)": Similar-sized seedlings were placed in 8692 nutrient solution amended with 0, 2.0, 8.0, and 16.0 mg Cr/L for 2-day exposure; (2) "Pro + Cr(VI)": After a 12-h pretreatment with Pro solution (1 mM), seedlings of the same age and size were placed in the recommended nutrition solution containing 0, 2.0, 8.0, and 16.0 mg Cr/L for a for 2-day exposure. The three concentrations, i.e., 2.0, 8.0, and 16.0 mg Cr/L, used in the study represent three effective concentrations (EC) with the growth inhibition of rice seedlings by 25%, 50%, and 75%, respectively, as determined in our previous study [13]. Potassium chromate (K_2CrO_4) of analytical grade was employed as the source of Cr(VI) for treatment purposes. Each flask was wrapped in an aluminum sheet to reduce evaporation and suppress the formation of algae.

Relative growth rate

Rice seedlings were weighed before the application of treatments and again at the termination of exposure period. The following equation was used to estimate the relative growth rate (RGR, %):

$$\mathrm{RGR} = \frac{M_F - M_I}{M_F} \times 100\%$$

where M_I and M_F are the initial and final weight of seedlings (g), respectively.

Measurement of pectin and PME activity

After being exposed to Cr(VI) treatments, the seedlings were harvested and washed with deionized water. 0.1 g of fresh roots and shoots were collected from treated

and untreated plants and subjected to pectin measurement. The method is described with details in Supporting information M1. The absorbance was monitored at 450 nm and pectin concentration was quantified using a standard galactose curve with known concentrations and expressed as microgram per gram FW.

For PME activity, 0.1 g of fresh roots and shoots were crushed in liquid nitrogen and processed further, as mentioned in Supporting information M1. The color change was measured spectrophotometrically at 525 nm within 2 min and was used to calculate the PME activity.

Measurement of total Cr content

After the treatments, the total Cr concentration in the rice plants was measured by employing the method of Pan et al. [14]. Seedlings from treated and untreated groups were placed in a cleaning solution containing $CaCl_2$ (1 mM) and MES-Tris buffer (2 mM, pH 6.0) to remove Cr ions adhered to the root surface. To digest the oven-dried plant samples (which had been dried for 48 h at 96 °C), a digestion solution comprised of a 4:1 mixture of HNO_3 and $HClO_4$ was used. Finally, the Cr content (µg/g DW) in rice tissues was determined by ICA–AES (PerkinElmer Optima 700DV). All the chemicals used in this study were of analytical grade.

RNA extraction and RT-qPCR analysis

RNA isolation and purification were carried out according to the procedure of Pan et al. [15]. Detailed procedure is given in Supporting information M1. Nine genes from the rice PME family, namely *OsPME6, OsPME7, OsPME9, OsPME10, OsPME13, OsPME23, OsPME24, OsPME25,* and *OsPME31* were assessed by RT-qPCR. Table S2 in the Supplementary Materials lists the primer sequences utilized in this experiment. RT-qPCR conditions are provided in Supporting information M1. Each targeted gene's relative expression was determined using the standard $2^{-\Delta\Delta CT}$ method.

The binding sites of selected PME genes to Cr(VI) ligand

The specific binding sites of the selected PME proteins to the Cr(VI) ligand were predicted using LeDock (v. 1.0). At uniprot (https://www.uniprot.org/), all PME 3D structures (SWISS–MODEL ID poc432) and its active sites were searched. While Pymol (V.3.2.0) was employed to predict the active binding sites of PME.

Calculation of gene expression variation factors (GEVFs)

By comparing the fold change in gene expression of the chosen PME isogenes in "Cr(VI)" treatments to "Pro + Cr(VI)" treatments, the *GEVFs* (%) were found:

$$\mathrm{GEVFs} = \frac{\mathrm{FC}_{(\mathrm{Pro+Cr})} - \mathrm{FC}_{(\mathrm{Cr})}}{\mathrm{FC}_{(\mathrm{Cr})}} \times 100\%$$

wherein $FC_{(Cr)}$ and $FC_{(Pro+Cr)}$ indicate the fold variations in PME isogenes' expression from "Cr(VI)" and "Pro + Cr(VI)" treatments, respectively. The *GEVF* threshold was established at > 25% or < − 25%, suggesting either gene promotion or repression, and indicating a statistically significant difference ($P < 0.05$).

Data analysis

For each experiment, four separate biological replicates were performed, and the mean ± standard deviation (SD) was used to present the data. Tukey multiple comparison tests at a significance threshold of $P < 0.05$ were employed to assess the statistical significance of the variation between the control and treatment groups.

Results

Relative growth rate

The relative growth rate of rice seedlings under "Cr(VI)" treatments was significantly reduced ($P < 0.05$) at all the tested concentrations compared to the "Pro + Cr(VI)" treatments, with the exception of the highest concentration tested (Fig. S1). This reduction in relative growth rate highlights the detrimental impact of Cr(VI) on rice seedling growth. In contrast, the relative growth rate of rice seedlings treated with "Pro + Cr(VI)" indicates a positive effect of exogenous proline on plant growth, suggesting that proline mitigates the adverse effects of Cr(VI) and promotes growth under stress conditions.

Accumulation of Cr in rice plants

Rice seedlings were analyzed for Cr accumulation under 0, 2.0, 8.0, and 16.0 mg/L of Cr, wherein the Cr concentration in untreated seedlings was below the detection limit, while in treated seedlings, the concentration in both shoots and roots enhanced with the rise in Cr doses. A significant ($P < 0.05$) difference was found in Cr accumulation between "Cr(VI)" and "Pro + Cr(VI)" treatments in both roots and shoots at all the ECs of Cr (Fig. 1). In roots, Pro treatment markedly ($P < 0.05$) enhanced Cr accumulation in comparison with "Cr(VI)" treatments alone. Interestingly, Pro treatment greatly ($P < 0.05$) reduced Cr accumulation in shoots compared with "Cr(VI)" treatment alone.

Pectin response to Cr exposure

The pectin content in "Cr(VI)"- and "Pro + Cr(VI)"-treated rice plants was assessed to determine pectin response to Cr(VI) exposure. A great difference was found between the treatments of "Cr(VI)" and "Pro + Cr(VI)" with respect to pectin concentration

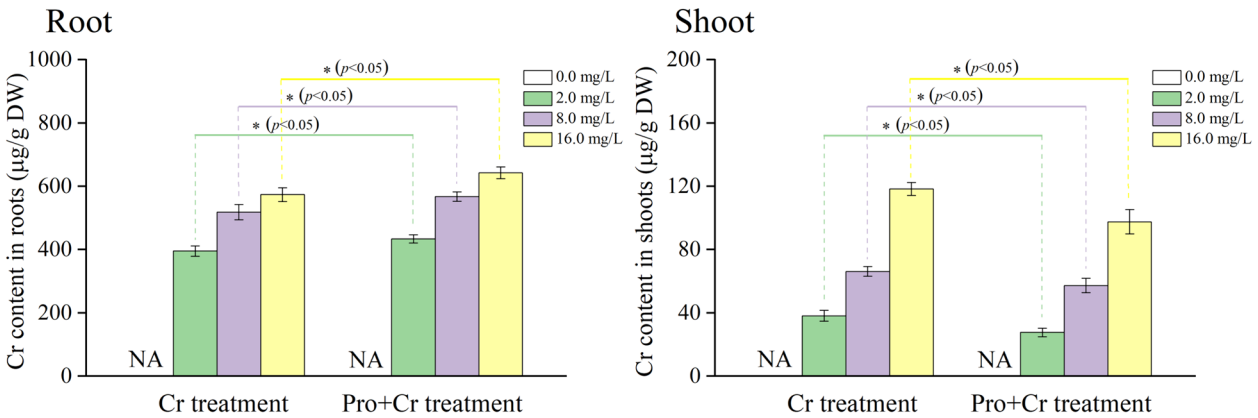


Fig. 1 Total Cr content (µg/g DW) in roots and shoots of rice seedlings under Cr(VI) stress in the presence or absence of exogenous Pro. Values are the mean of four independent biological replicates ± standard deviation. NA denotes concentrations below the limit of Cr detection. The asterisk (*) refers to the significant difference between the "Cr(VI)" treatments and "Pro + Cr(VI)" treatments

in both root and shoot tissues of rice seedlings (Fig. 2). Although pectin content rose in rice roots at the initial concentration, i.e., 2 mg Cr/L; however, at higher doses, i.e., 8, and 16 mg Cr/L, it remained unaffected. Compared with "Cr(VI)" treatments, Pro application with Cr(VI) significantly ($P < 0.05$) increased pectin concentration in rice roots except at 2 mg Cr/L ($P > 0.05$). In shoots, Cr(VI) did not affect pectin content, while Pro treatment significantly ($P < 0.05$) enhanced pectin content at 0 and 2.0 mg Cr/L, but insignificantly ($P > 0.05$) at 8.0, and 16.0 mg Cr/L.

Responses of PME activity to Cr exposure

The exogenous Pro application significantly increased the activity of PME in rice tissues in response to Cr(VI) exposure (Fig. 3). PME activity in roots was repressed by all three ECs of Cr(VI) in comparison to the control group. However, its activity was significantly ($P < 0.05$) induced by Pro application with Cr(VI), while the Pro-treated control (Pro + 0 mg Cr/L) remained similar to the empty control (0 mg Cr/L). The increase in Cr concentration changed the PME activity in rice shoots exposed to Cr(VI). Applying Pro to "Cr(VI)" treatments caused a substantial ($P < 0.05$) increase in PME activity, except 8.0 mg Cr/L ($P > 0.05$).

Identification of PME genes involved in pectin demethylation

Pectin methylesterase is a ubiquitous enzyme that demethylases pectin, thereby changing its structure [5]. This alteration is primarily linked to the binding and accumulation of HM ions within cell walls [7]. Following BLAST-P searches, 35 isogenes were found in the rice PME genome family from the three rice databases from RGAP (http://rice.plantbiology.msu.edu/analyses_search_blast.shtml), CRTC (http://www.ricedata.cn/gene/index.

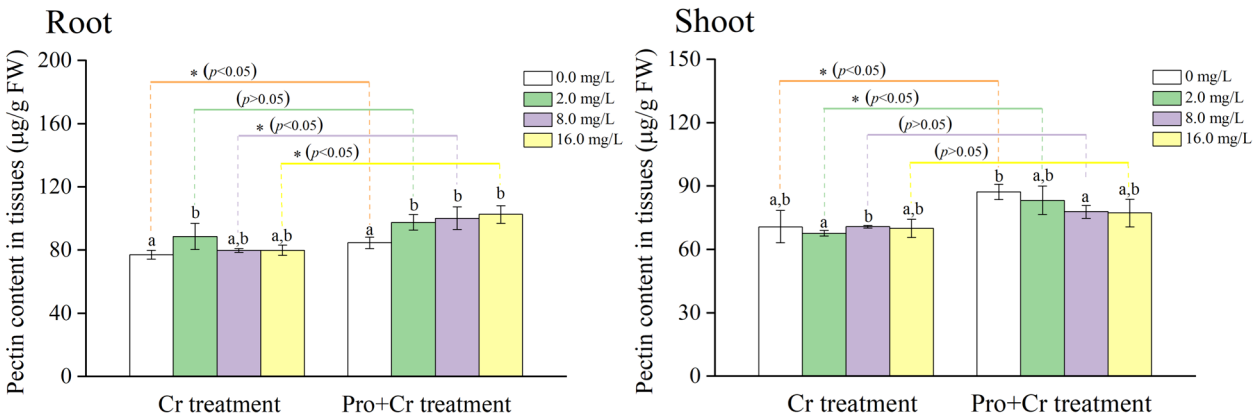


Fig. 2 Pectin content (µg/g FW) in roots and shoots of rice seedlings under Cr(VI) stress in the presence or absence of exogenous Pro. Values are the mean of four independent biological replicates ± standard deviation. The asterisk (*) refers to the significant difference between the "Cr(VI)" treatments and "Pro + Cr(VI)" treatments. Different letters refer to the significant difference between the treated seedlings and the control ($p < 0.05$)

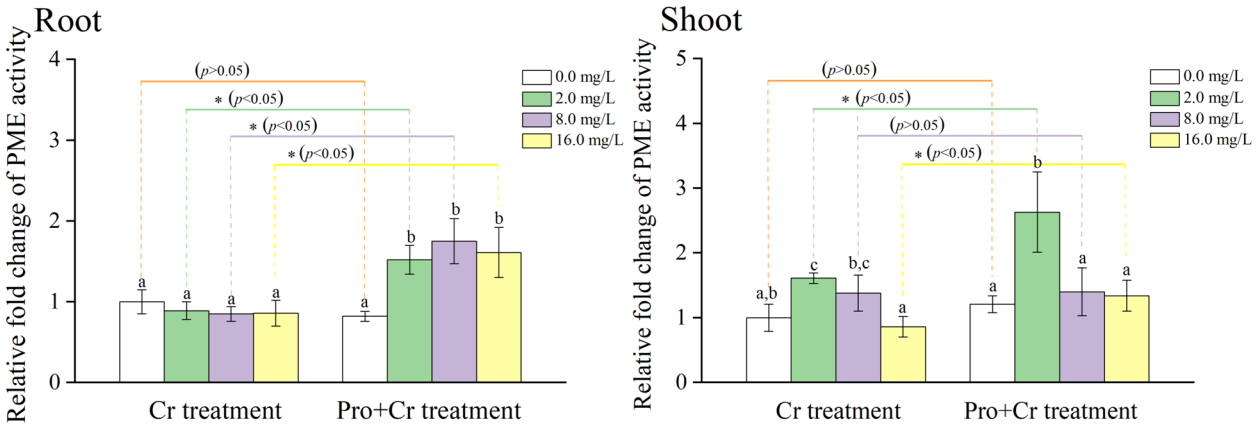


Fig. 3 Response of PME activity in roots and shoots under Cr(VI) stress in the presence or absence of exogenous Pro. Values are the mean of four independent biological replicates ± standard deviation. The asterisk (*) refers to the significant difference between the "Cr(VI)" treatments and "Pro + Cr(VI)" treatments. Different letters refer to the significant difference between the treated seedlings and the control ($p < 0.05$)

htm), and RAP-DB (http://rapdb.dna.affrc.go.jp/). These genes were identified in *Arabidopsis thaliana* based on their individual sequences from the PME family. Among these isogenes, only 22 genes clustered in Clade 1 contain the PME domain (Fig. 4). Phylogenetic analysis showed that most genes grouped in Clade 1 are activated in heat tolerance, callus formation, pollen tube growth, and plant–pathogen interaction [19–21], wherein only *OsPME9* shares the closest phylogenetic relationship with *AtPME17* and *AtPM35*, which are defined in Arabidopsis functioning in demethylation of pectin [22, 23]. The functions of other genes, i.e., *OsPME6, OsPME7, OsPME10, OsPME13, OsPME23, OsPME24, OsPME25*, and *OsPME31* grouped in Clade 1, are not defined yet.

Gene expression of PME genes

The expression pattern of the nine genes was evaluated in all treatments of Cr with and without Pro application. When compared to "Cr(VI)" treatments, exogenous Pro had a beneficial influence on the expression of *OsPME7, OsPME9, OsPME24*, and *OsPME25* genes in the roots of rice plants, but the remaining genes were down-regulated (Fig. 5). Similar beneficial effects of exogenous Pro on gene expression were observed in the case of shoots between the "Cr(VI)" and "Pro + Cr(VI)" treatments, where increased gene expression was observed.

The gene expression variation factors (GEVFs)

The effect of exogenous Pro application on the transcription of genes involved in pectin demethylation under different Cr(VI) treatments was estimated using *GEVFs* (Fig. 6), and Supplementary Tables S3 (roots) and S4 (shoots) display their detailed information. According to the *GEVFs* data, the promoting genes (*GEVFs* > 25%) in rice roots were assigned to *OsPME7, OsPME9, OsPME24,* and *OsPME25* at various Cr(VI) treatment concentrations (Fig. 6a). The Venn diagram suggests that only *OsPME7* and *OsPME9* are the mutual "promoting genes" observed under the three ECs of Cr(VI) in rice roots, wherein *OsPME25* was the "promoting genes" at 8 and 16 mg Cr/L (Fig. 6b). These analysis suggests that the PME activity is more significantly impacted by Pro application via *OsPME7* and *OsPME9* than other OsPME isogenes of roots. For instance, the scores for *OsPME7* in rice roots were 83.61, 33.02264, and 185.99, while for *OsPME9,* they were 170.99, 926.40, and 2268.58 at concentrations of 2.0, 8.0, and 16.0 mg Cr/L, respectively, indicating higher expression levels compared to all other selected genes. In rice shoots, the higher *GEVF* values were assigned to *OsPME7, OsPME9, OsPME24*, and *OsPME25* at all the ECs of Cr (Fig. 6c). The Venn diagram showed that *OsPME7, OsPME9, OsPME24*, and *OsPME25* in shoots are the mutual "promoting genes" observed at all the three concentrations of Cr(VI) (Fig. 6d).

Affinity potential of PME for Cr(VI) ligand

To find out the binding sites of PME proteins with the Cr(VI) ligands, a molecular docking technique was used. The proteins of OsPME isoforms that were identified in the previous section (3.4) were used for molecular docking, except OsPME6, OsPME10, OsPME13, OsPME23, and OsPME31, which show negative responses to Cr(VI) with the application of exogenous Pro. Figure 7 shows the interaction sites of OsPME7, OsPME9, OsPME24, and OsPME25 with Cr(VI) ligands. We observed that the amino acid residues, Gln 356, Arg-398, and Glu-406, show interactions with the Cr(VI) ligands in the case of

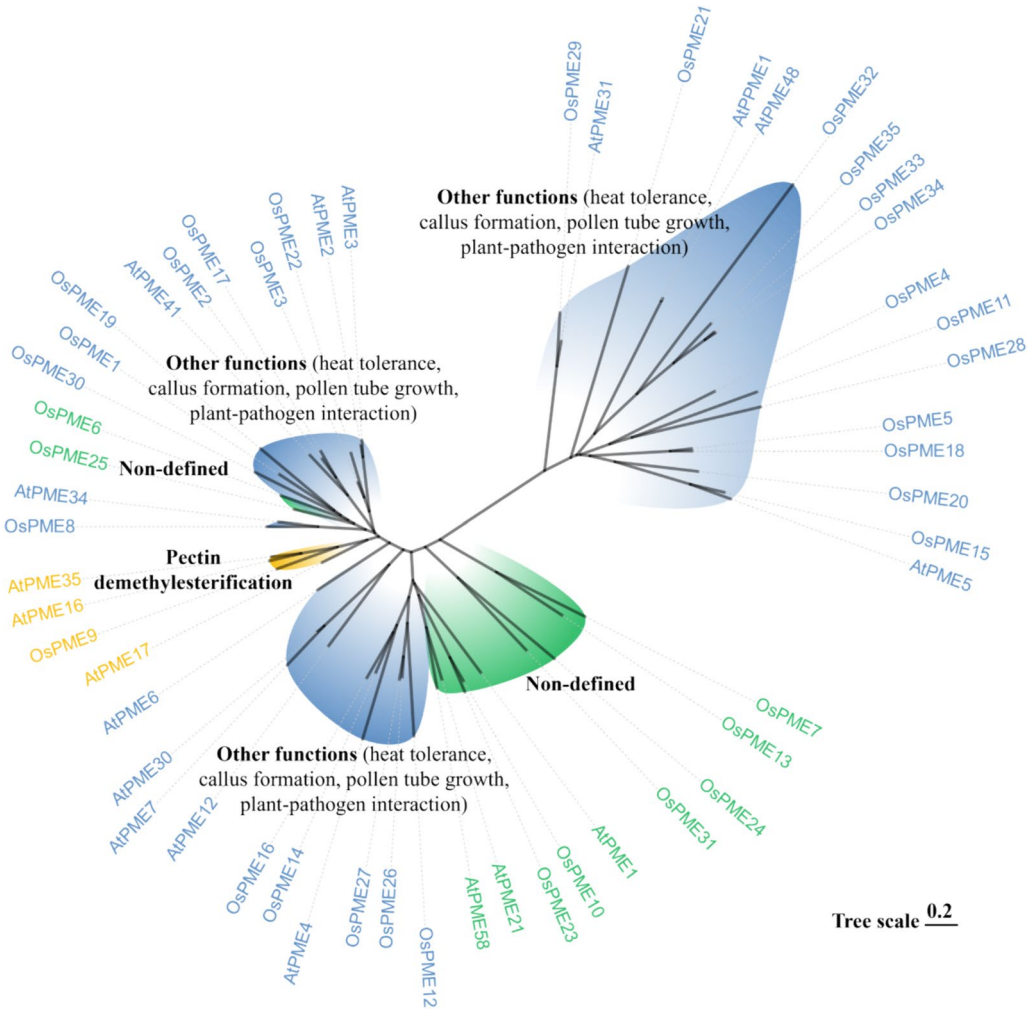


Fig. 4 Phylogenetic relationships of the PME sequences from rice and *Arabidopsis* by MEGA7.0.18 program using neighbor-joining methods with 1000 bootstrap replicates. The PME genes marked in different colors refer to the genes with different functions. Blue color marked refers to the genes functioning in heat tolerance, callus formation, pollen tube growth, and plant–pathogen interaction, yellow color marked refers to the genes activated in pectin demethylesterification, and the functions of the genes marked in green color were not defined yet

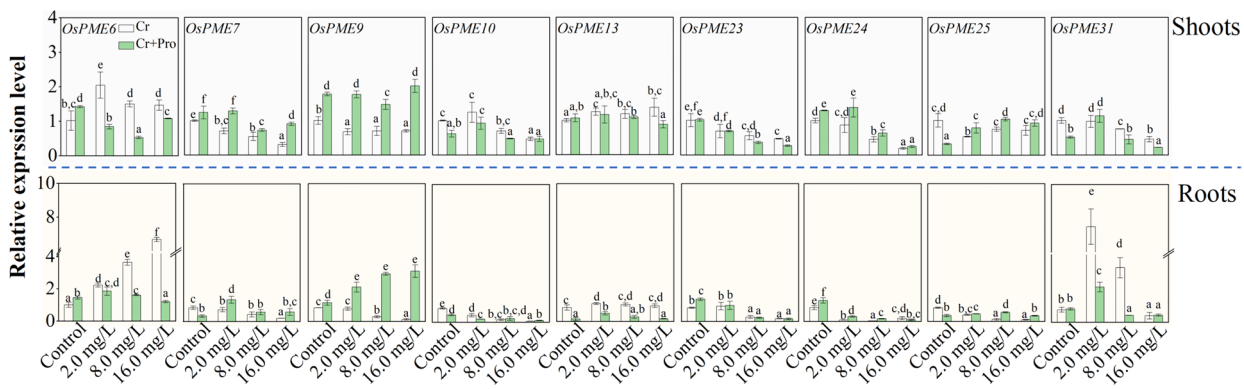


Fig. 5 Relative expression of PME genes in rice tissues under Cr(VI) stress in the presence or absence of Pro. Values are the mean of four independent biological replicates ± standard deviation. The asterisk (*) refers to the significant difference between "Cr(VI)" treatments and "Pro + Cr(VI)" treatments

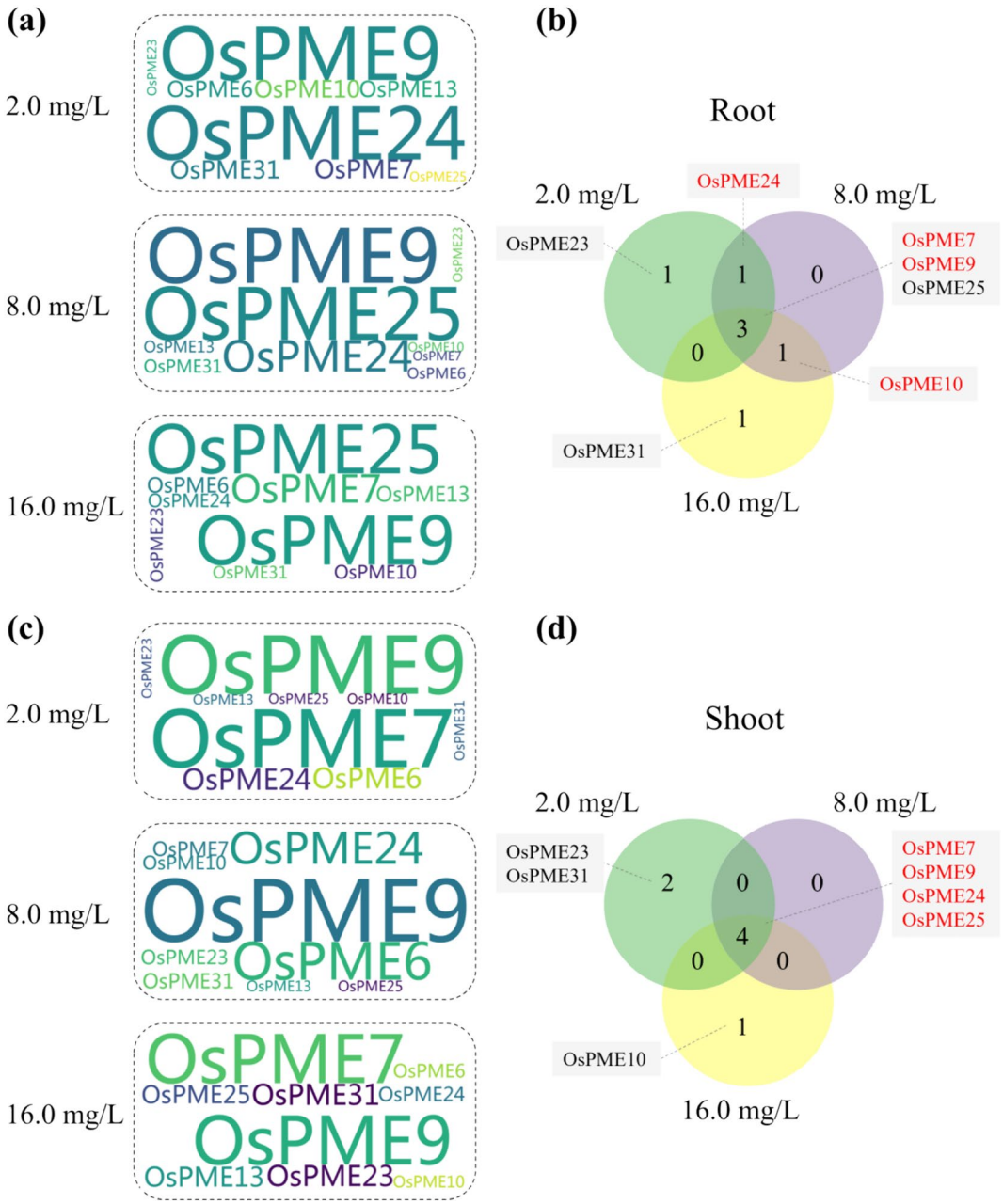


Fig. 6 Gene expression variation factors (*GEVFs*) between "Cr(VI)" treatments and "Pro + Cr(VI)" treatments in rice seedlings. The *GEVFs* in root (**a**) and shoots (**c**) of rice seedlings at three different concentrations of Cr(VI) (* the larger size of gene symbol indicates a higher value of *GEVFs*); The Venn diagram represents all positive values of *GEVFs* in roots (**b**) and shoots (**d**). The red color genes marked in (**b**) and (**d**) refer to the "promoting genes" in rice tissues

OsPME7. While, OsPME9 has a single amino acid residue, Gly 361 that can interact with Cr(VI) ligands. Moreover, Cr(VI) ligands showed substantial interactions with Asp 99, Glu 97, and Trp 121 residue of OsPME24; and Gln 407, Gln 461, and Arg 461 of OsPME25 (Table 1). These binding sites from each PME isoform with Cr(VI) ligands suggest their substantial binding potential for Cr(VI) ligands may cause different effects on the PME activity and subsequently alter the demethylation process of pectin. It is interesting to notice that OsPME9 only has one single binding site with Cr(VI) ligands, and the other three PME proteins have three binding sites. Herein, additional estimation of the affinity potential between the ligand and the receptor (protein) was

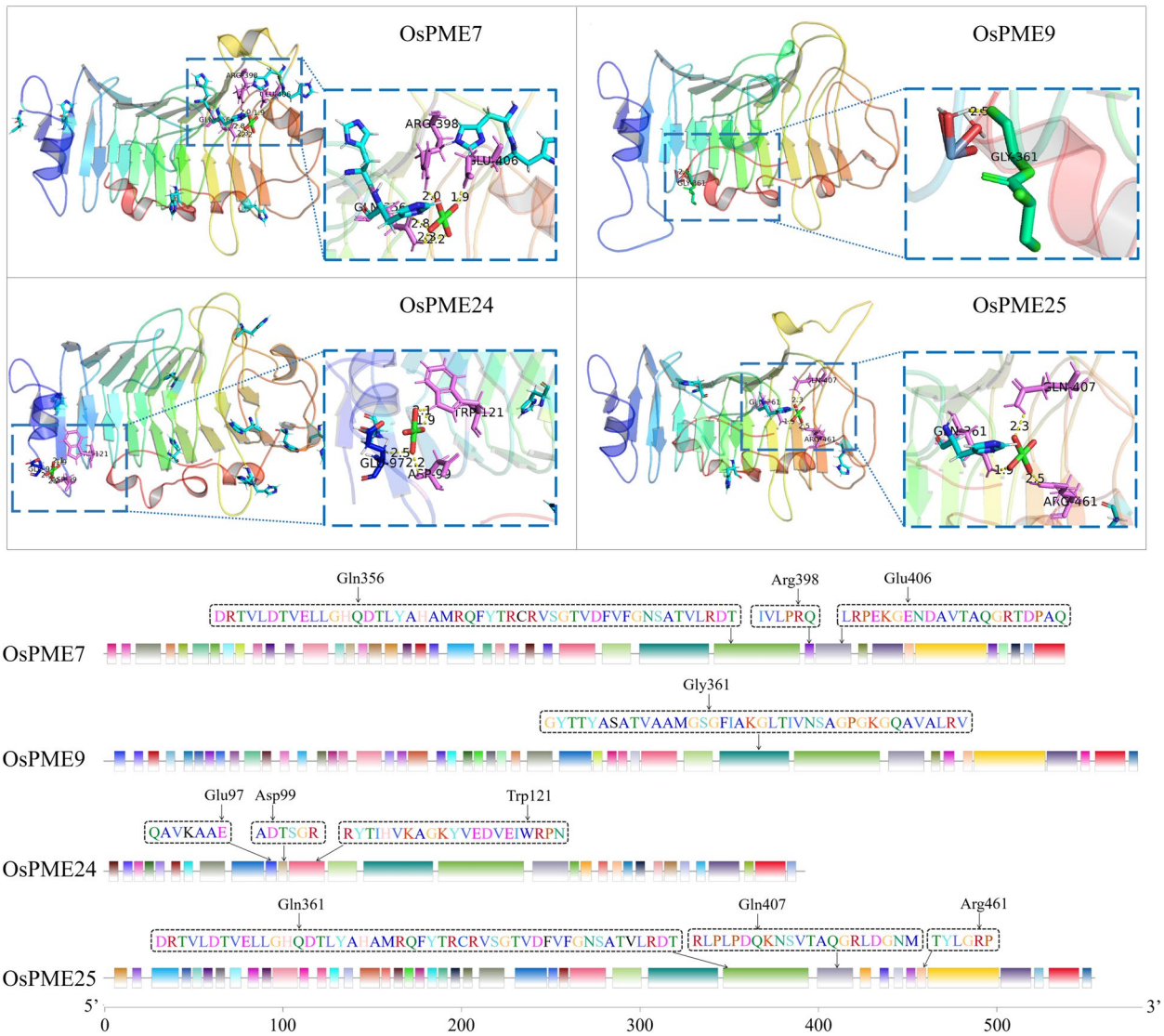


Fig. 7 Binding sites of selected PME proteins with Cr(VI) ligand and the protein sequence of the conserved motifs of the possible binding sites

Table 1 Detailed information of possible binding sites of isogenes with Cr(VI) ligand

Gene name	Binding site	Total score
OsPME7	Gln356, Arg398, Glu406	− 3.4211
OsPME9	Gly361	− 3.6824
OsPME24	Asp99, Glu97, Trp121	− 3.4272
OsPME25	Gln407, Gln361, Arg461	− 3.3132

calculated by a scoring function carried out by the MOE (Molecular Operating Environment 2019.0102) online program, wherein the elements of the contribution of translational/rotational entropy, electrostatic interaction, solvent effect, van der Waals effect, and solvent exposure area were considered during the MOE score estimation, and the greater absolute value of total score indicates the stronger interaction between the ligand and the receptor. The scores of the affinity potential between selected PME protein and Cr(VI) ligands are given in Table 1.

Discussion

The uptake of Cr(VI) by plants primarily occurs through the symplastic pathway, potentially leading to toxicity in plant cells [24]. This toxicity subsequently reduces plant growth, as demonstrated by the significant reduction in relative growth rate of rice seedlings under Cr(VI) treatments (Fig. S1). Among several response mechanisms,

sequestration and compartmentalization of HMs are the crucial mechanisms to reduce their toxicity in plants by keeping them away from the vital cellular organelles [25]. The cell wall in this regard is an extracellular matrix that accumulates HMs predominantly to reduce its induction into the cytoplasm and other organelles including plasma membrane, mitochondria, chloroplasts, endoplasmic reticulum, and nucleus. In addition, plants struggle to accumulate a higher quantity of HMs in roots to limit their translocation into shoots [26]. For instance, the accumulation of Cr in dried mung bean (*Vigna radiata*) plants was 3.83, 2.23, 1.21, and 0.71 mg/g in roots, stems, leaves, and seeds, respectively [27]. Proline, as a HMs chelator, enhances Cr accumulation and tolerance against these metals in plants. In a case, exogenous Pro enhanced plant growth and expression of transcription factors in rice tissues, which subsequently lowered the negative effects of Cr(VI) in rice plants [15]. In our findings, exogenous Pro significantly ($P < 0.05$) enhanced Cr accumulation in roots but decreased ($P < 0.05$) in shoots of rice seedlings surprisingly (Fig. 1). Since, the leaves and stems are more susceptible to HMs adversity, its accumulation in roots serves as a general defense mechanism to lessen metal ion translocation into them [28]. In fact, a plant's root system is its primary organ in direct contact with soil polluted with HMs, and it is crucial to the absorption, accumulation, and transport of HMs, including Cr [26].

Since the cell wall is a cell's first protective line, accumulates higher concentrations of HMs followed by other organelles and cytoplasm [14, 29]. *Cosmos bipinnatus* accumulated most of the Cr in their root's cell walls, and changes in pectin content significantly affected the concentration of Cr accumulation in these cell walls [30]. Herein, the pectin concentration in rice tissues was independent of Cr(VI) doses supplied, while Pro application significantly increased the pectin concentration in Cr(VI)-treated rice seedlings (Fig. 2). It suggests that an increase in pectin content under "Pro + Cr(VI)" treatments lead to an increase fixing of Cr(VI) ions in roots cell walls. Pectin and cellulose are the major components of cell walls that actively participate in Cr binding through the –COOH, –OH, and –SH groups [2, 29]. Three main domains are recognized in pectin, i.e., rhamnogalacturonan-I, rhamnogalacturonan-II, and homogalacturonan, in which homogalacturonan is the main (65%) pectin domain responsible for binding metal cations [2]. The homogalacturonan polysaccharide is further composed of a high methyl-esterified form of α-1,4-linked D-galacturonic acid residues that can be de-esterified by PME [31]. The activity of PME is directly associated with pectin demethylation, which removes methyl groups from the pectin and exposes carboxyl groups with higher affinity for HMs including Cr [5]. Herein, Cr(VI) exposure repressed PME activities in rice roots, while exogenous Pro significantly increased PME activities (Fig. 3). It indicates that Cr(VI) acts as an inhibitor of PME enzymes, which reduces their activity and subsequently reduces Cr(VI) adsorption to the cell wall. In contrast, Pro does not allow or reduce Cr(VI) binding with PME which leads to a normal activity of PME and subsequently higher levels of pectin de-esterification and binding of Cr(VI) to the cell wall. Although a slight increase in PME activity was also recorded in rice shoots with Pro application, its effects were more obvious in roots, which explains why root tissues accumulated a higher concentration of Cr(VI). Furthermore, these findings demonstrate the positive impact of exogenous Pro on the pectin content and PME activity of the cell wall during Cr(VI) exposure. In addition, exogenous proline leads to an increase in plant biomass, as indicated by the relative growth rate of rice seedlings under "Pro + Cr(VI)" treatment being significantly greater than under "Cr(VI)" treatments (Fig. S1).

To gain insights into the mechanism of how Cr(VI) exposure acts as a reducer of PME activities in rice roots, while exogenous Pro significantly increased PME activities, it is important to find the key genes that regulate the PME activity. In this regard, phylogenetic analysis revealed that only *OsPME9* shares the closest phylogenetic relationships with *AtPME17* and *AtPM35* (Fig. 4), while demethylesterification of pectin by these genes is well-studied in Arabidopsis [22, 23]. However, the function other genes, i.e., *OsPME6, OsPME7, OsPME10, OsPME13, OsPME23, OsPME24, OsPME25,* and *OsPME31* grouped in Clade 1, are not defined yet. During the PCR test, five of them (*OsPME6*, *OsPME10*, *OsPME13, OsPME23*, and *OsPME31*) showed a negative response to exogenous Pro application, suggesting that they are not involved in the demethylation of pectin catalyzed by PME (Fig. 5). Furthermore, the binding sites of OsPME7, OsPME9, OsPME24, and OsPME25 with the Cr(VI) ligand were identified via molecular docking technique, suggesting the binding affinity of Cr(VI) ions with the amino acid residues of PME proteins (Fig. 7).

The molecular docking technique has previously been used to find the interactions of pectin with proteins [32]. Several amino acid residues of the PME proteins that can interact with the Cr ligand and subsequently bind and reduce its activity were observed. For instance, OsPME7, OsPME24, and OsPME25 each have three binding sites; OsPME9 has only a single binding site for Cr binding. The number of binding sites of each PME protein with the Cr(VI) ligand reveals a possible effect on PME activity and subsequently alters pectin adsorption in the cell wall. In

this study, a scoring function was used to estimate the affinity potential between the Cr(VI) and PME isoprotein selected. The results from this calculation reveal that the difference in total scores was marginal (Mean: − 3.4610, S.D. 0.16, $n=4$) between four PME proteins, suggesting that only using the result of scoring function to estimate the affinity potential between the Cr(VI) and PME isoprotein is insufficient. However, integrating the scoring function analysis with the *GEVF* estimation, we observed that among these common "promoting genes" obtained from the three ECs of Cr(VI), *OsPME7* and *OsPME9* had much higher *GEVF* values than *OsPME24* and *OsPME25*. Also, the *GEVF* values *of OsPME7* and *OsPME9* showed positive responses to Cr(VI) concentrations from both roots and shoots in the presence of Pro, while the changes of *GEVF* values *of OsPME24* (Mean: 42.8, S.D. 15.3, $n=3$) and *OsPME25* (Mean: 38.4, S.D. 8.61, $n=3$) in shoots was independent of Cr(VI) concentrations, suggesting that the effect of exogenous Pro was remarkable under Cr(VI) exposure, and the expression of *OsPME7* and *OsPME9* was much more sensitive to the application of exogenous Pro than *OsPME24* and *OsPME25*. Therefore, it affirms that OsPME7 and OsPME9 have a higher weightage in PME activity functioning in pectin demethylation that increases the abundance of Cr(VI) adsorbing functional groups, i.e., –COOH, –OH, and –SH [33] and consequently Cr(VI) adsorption in the cell wall of rice tissues. These results reveal that Pro improves the PME activity and subsequent demethylation of pectin and adsorption of Cr(VI) ligand via the main regulation of *OsPME7* and *OsPME9*. Pro are important amino acids that are crucial for the normal activity of a protein [34], and Cr(VI) ligand binding with PME might reduce its activity by denaturing some proteins, which may be rescued by Pro application via enhancing the expression of crucial players in their activity. In a recent study, Pro induced the expression of several transcription factors that regulate Cr(VI) stress in rice plants [35]. These results were further confirmed by the *GEVFs* among the "Cr(VI)" and "Pro + Cr(VI)" treatments, where *OsPME7* and *OsPME9* received the higher positive *GEVF* values than the other two genes, i.e., *OsPME24* and *OsPME25,* in both roots and shoots tissue of rice (Fig. 6; Tables S3 and 4). Analysis of *GEVFs* indicates that Pro has a beneficial impact on several genes that were tested, however, *OsPME7* and *OsPME9* were found to have a more significant role in PME regulation among the rice seedlings' roots and shoots across all the ECs of Cr(VI). These results are positively correlated with pectin content and PME activity results of the cell wall.

Conclusions

Hexavalent chromium treatments reduced the PME activity, which subsequently reduced the demethylation of pectin and Cr(VI) adsorption in the cell walls of rice plants. Integration of the scoring function analysis with the *GEVF* estimation, several amino acid residues in PME proteins can bind Cr(VI) ligand, which leads to repression of its activity, wherein *OsPME7* and *OsPME9* were found to have a more significant role in PME activity than *OsPME24* and *OsPME25* among the rice seedlings' roots and shoots across all the ECs of Cr(VI). Application of exogenous Pro increased PME activity and pectin concentration by enhancing the expression of PME-associated genes. Based on these findings, we suggest to further investigate the specific amino acid residues in PME proteins that bind Cr(VI) ligands and repress PME activity. Exploring the roles of *OsPME7* and *OsPME9* in PME activity under Cr(VI) stress in more detail would be beneficiary that why these genes played a significant role in PME activity.

Supplementary Information

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Additional file 1.

Author contributions

UA was responsible for writing original draft preparation, investigation, and data analysis; LYJ was responsible for investigation, data analysis, visualization, and funding acquisition. TP was responsible for investigation. YXZ was responsible for conceptualization, methodology, supervision, writing—reviewing, and funding acquisition. All the authors contributed to the final review of the manuscript.

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Availability of data and materials

The data used and/or analyzed in this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]College of Environmental Science and Engineering, Guilin University of Technology, Guilin 541004, People's Republic of China.

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