Physio-Biochemical Characteristics of Root Cell Wall in Salinity Tolerance Mechanisms in Wheat

（コムギの耐塩性機構としての根細胞壁の生理生化学的特性）

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Chapter 1. General introduction

Wheat (*Triticum aestivum* L.) is an important food crop grown worldwide, accounting for approximately 25% of the world's staple food (Pocketbook, 2018). Salinity is one of the major factors that affect crop growth, affecting large terrestrial areas of the world (Zhu 2001). Although the salinity tolerance of wheat is strong (Shabala 2017), the increase in salt concentration reduces wheat yield (Munns et al. 2006). Overcoming yield losses in crops under salinity is important to study.

Studies of the salt tolerance mechanisms of plants have mainly focused on osmotic adjustment, membrane function, and gene expression (Shabala 2017). However, the function of cell wall in plant salt tolerance and its responses under saline conditions have been rarely investigated (Deinlein et al., 2014). Especially in plant roots, the root cell walls act as the first line, directly interacting with salt. Studies showed that pectin modification (Yan et al. 2018), cellulose synthesis (Zhang et al. 2016), and hemicellulose synthesis (Li et al. 2013) were involved in response to salt stress. Changes in cell wall properties and physical characteristics under salinity stress are of primary importance to the plant's tolerance to salinity (Byrt et al., 2018). Particularly in the root tips, the cell wall composition is involved in cell elongation, thereby affecting root growth.

1.1 Wheat growth under salinity

Electrical conductivity (EC) of the saturation paste extract exceeding 4 ds/m (equivalent to 40 mM NaCl) is defined as saline soil (Shabala 2017). Because the salt concentration experienced by roots can be several times higher than that in the saturation extract, a soil with an EC of 4 ds/m often has a concentration of 80–100 mM NaCl (Shabala 2017). The excessive salt cause series of detrimental effects on plant, including osmotic stress, ionic stress, oxidative

stress, plant growth, photosynthesis, metabolic disturbances, and ion toxicity (Shabala 2017). Among these effects, the most significant and oblivious one is the plants growth inhibition.

The detrimental effects of salinity contain two stages. The first stage is an osmotic-stress stage. The second stage is ion toxicity stage caused by accumulations of Na^+ under salinity stress. During the first stage, the plant growth is sensitive to the salinity stress. The roots firstly stop growing (quiescent phase) after the salinity stress perception (Fig. 1-1; van Zelm et al. 2020). Thereafter, the growth rate partially recovers (recovery phase). The recovery rate of growth depends on the plant organ, local sodium concentrations, and salt sensitivity (Julkowska and Testerink 2015). For example, in *Arabidopsis*, the growth rate is recovered by 50% in primary root, while the growth rate is 10% in the lateral root (Duan et al. 2013).

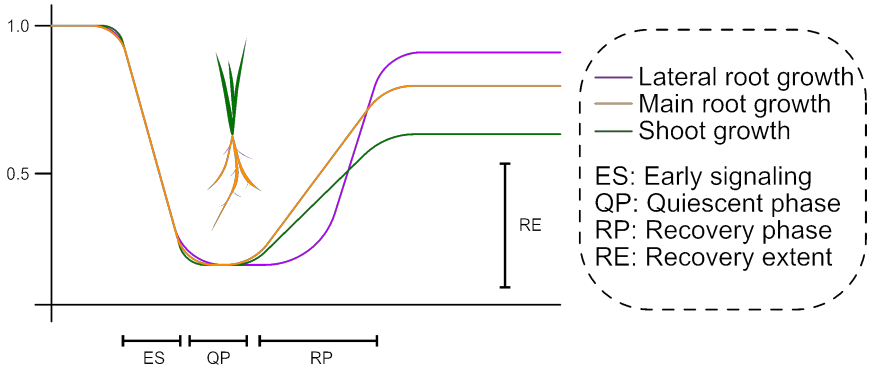

Figure 1-1 Phases of root growth in response to salinity stress. Plant first decreases growth rate during early signalling phase, and then stops growth (quiescent phase). After 24–48 h, the growth is partially recovered (recovery phase). The recovered growth (recovery extent) rate under salinity stress is lower than that under normal condition. The growth rates of organ are different under salinity stress. This schematic is cited from Julkowska and Testerink (2015).

1.2 Root growth in relation with salinity tolerance

Root growth maintenance is an important trait for plant to tolerate salinity stress (Mujeeb-Kazi et al. 2019). Especially during the seedling stage, root length under salinity condition is an important index to assess salt tolerance (Rahnama et al. 2011). High root growth rate leads to a robust root architecture, which increases the Na^+ sequestration capacity in root mature region and prevents the excessive salt accumulated in shoots (Munns et al. 2020). The high

root growth rate also optimized the root shoot ratio, resulting in an energy-usage balance between source and sink (Tyerman et al. 2019).

1.3 Cell wall compositions in response to salinity stress

Cell elongation is crucial for maintaining growth. In root tips, cell wall composition is involved in the cell elongation, thereby affecting root growth (Cosgrove 2018). As the outermost layer that constrains the cell growth, the cell wall is composed of relatively stiff cellulose microfibrils embedded in a hydrated matrix of pectin and hemicellulose (Fig. 1-2; Cosgrove, 2018). Pectin is associated with ion homeostasis (Koyama et al., 2001), hydraulic conductivity (McKenna et al., 2010), and cell wall expansion (Palin and Geitmann, 2012; Willats et al., 2001). Under salinity stress, plants modify composition and structure of the root cell walls (Byrt et al., 2018), affecting cell wall extensibility and cell elongation (Cosgrove, 2015; Tenhaken, 2015). Studies showed that Na^+ can also directly break the cell wall integrity under salinity stress (Feng et al. 2018). In response to salt stress, genes that regulate pectin modification (Yan et al. 2018), cellulose synthesis (Zhang et al. 2016), and hemicellulose synthesis (Li et al. 2013) are involved. These results indicate that changes in cell wall composition are indispensable for plants to cope with salt stress.

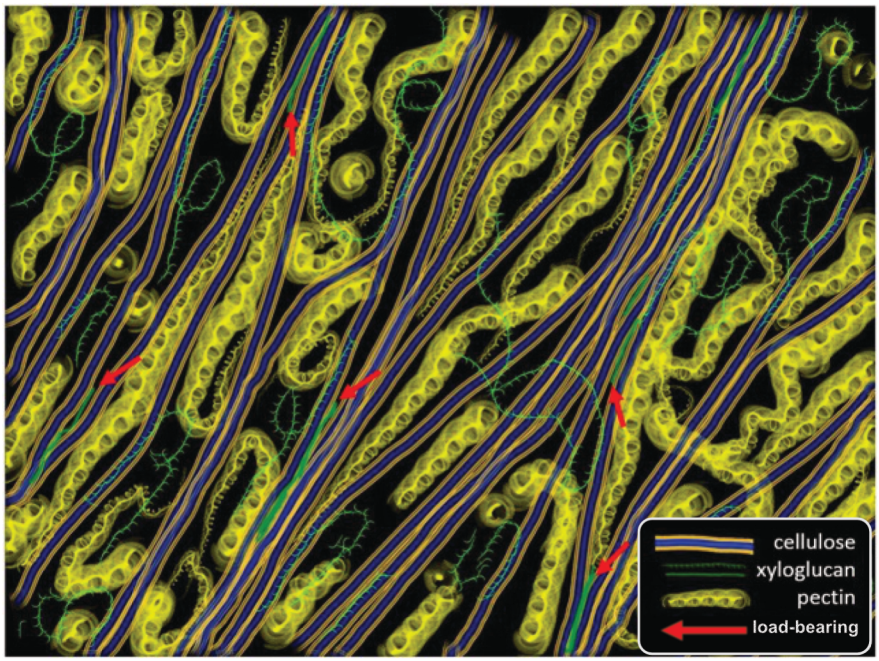

Figure 1-2 Conceptual depiction of structural compositions of primary cell walls. Cellulose microfibrils are represented as thick rods with hydrophobic (blue) and hydrophilic faces (orange). Hemicellulose (green) and pectin (yellow) fill the space between microfibrils and bind to the hydrophilic surfaces. The red arrows point to cellulose-xyloglucan-cellulose junctions. Cosgrove (2018) develops the 'hot-spot' model to explain the physical strength of cell wall. The cellulose-hemicellulose-cellulose conjunctions are the main load-bearing structure (red arrow; Cosgrove 2018).

The changes in composition of the cell wall affect not only the mechanical properties of the root, but also the chemical properties of cell wall (Byrt et al., 2018). The carboxyl groups of poly-galacturonic acid (PGA) and hydroxycinnamic acid are the chief cation-binding groups in pectin and hemicellulose (Fig. 1-3; Davis et al., 2003; Meychik et al., 2014; Pelloux et al., 2007). The ion transport from apoplast to plasma membrane can interact with these carboxyl groups (Meychik et al., 2014). Divalent cations can form the "egg-box" structure with pectin and hemicellulose contents in the cell wall (Grant et al., 1973), which maintains the integrity of cell wall structures (Byrt et al., 2018). Under salinity stress, sodium ions compete for the ion binding sites in the cell walls (Lutts et al., 2016) and undermine the pectin cross-links, resulting in a loss in cell integrity (Feng et al., 2018).

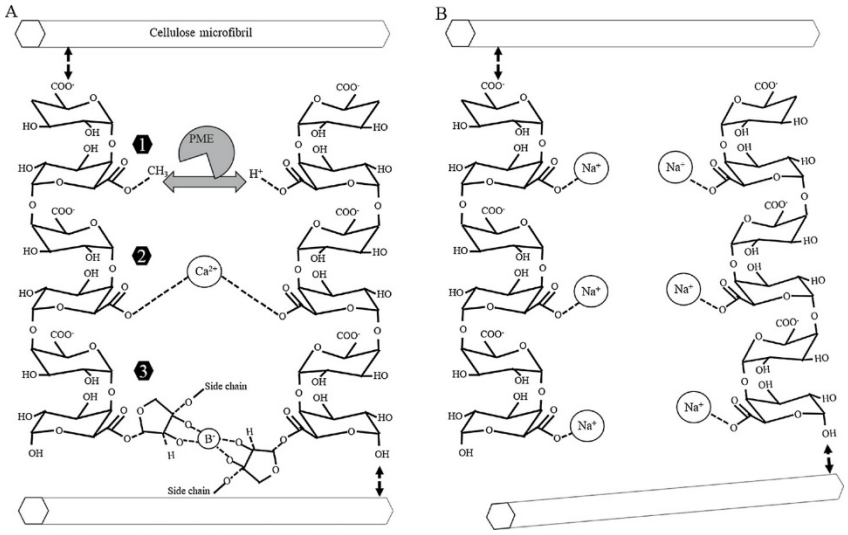

Figure 1-3 Egg-box model for how excess Na^+ might influence cell wall pectin properties. A. Cellulose microfibrils are linked by pectin, calcium, and boron. B. Na^+ binds to the polygalacturonic acid tails of cellulose microfibrils and breaks the interaction between cellulose microfibrils. This schematic is cited from Byrt et al. (2018).

1.4 Cell wall extension in response to salinity stress

Cell wall extension, composition, structure, and growth dynamics have been extensively reviewed by Cosgrove (2018). Pectin governs the cell wall extensibility by affecting the cell wall elasticity (Wolf and Greiner 2012). Recent studies have reported extensive pectin-cellulose interactions (Wang et al. 2015) and pectin-xylan links (Tan et al. 2013) in cell walls. Hemicellulose I is composed of long polysaccharide chains and is associated with cellulose microfibrils (Zhong and Lauchli 1993; Fry 2011). Hemicellulose II adheres to the surface of cellulose and forms cellulose-xyloglucan-cellulose conjunctions, which are major load bearing points for mechanical forces (Park and Cosgrove 2012; Zhang et al. 2014). Cellulose contributes to wall rigidity and mechanics (Zhang et al. 2014).

The primary cell wall behaves like a viscoelastic composite material that demonstrates a time-dependent extension under load and time-dependent shrinkage after stretching (Boudaoud 2010; Cosgrove 2018). A Kelvin-Voigt-Burgers model with four elastic (E_0, E_1, E_2, E_3) and four plastic (η_0, η_1, η_2, η_3) parameters effectively analyzed cell wall extension and shrinkage in the creep-extension analysis (Fig. 1-4; Tanimoto et al. 2000). Cell wall extension is partially

elastic and partially plastic (Boudaoud 2010), and the elastic and plastic parameters determine the elastic and plastic extension, respectively. Such extension (deformation) is a result of the polymeric nature of the cell wall, however, the mechanical properties are largely unknown (Cosgrove 2018).

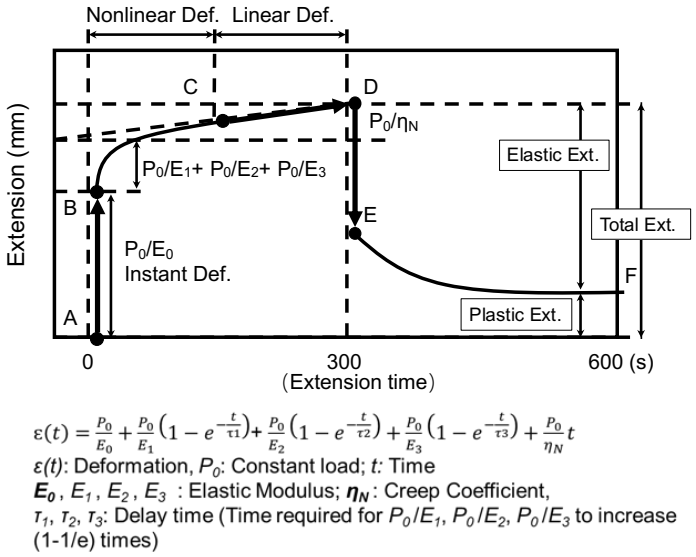

Figure 1-4. Schematic diagram of creep meter. E_0 and η_N are two most important parameters that represent cell wall extensibility. A typical creep extension curve during 5 min extension and 5 min shrinkage. Linear instantaneous deformation (A-B), nonlinear deformation (B-C) and final linear deformation (C-D) were simulated to the equation (B) and physical parameters of elastic moduli and viscosity coefficients were calculated. The schematic is modified from Tanimoto et al. (2000).

1.5 Expansin expression and apoplastic pH

The plant growth requires the initial cell wall loosening before the cell elongation. The cell loosening is elucidated as 'acidic growth theory', and further developed by Cosgrove et. al. (2000). The apoplastic pH activates the expansin, which then releases the binding sites of xyloglucan-cellulose conjunctions and loosens the cell wall. This process needs the interaction among apoplastic pH, cell wall properties and expansins (Fig. 1-5). Salinity stress changes the properties and structure of cell wall. Furthermore, studies showed the expression pattern of expansin was also changed under salinity stress (Han et al. 2019). In leaves, salinity stress alters the apoplastic pH, cell wall properties and expansins, resulting in significant growth

inhibition in response to salt (Geilfus 2017). However, there is limited information about the long-term response of root apoplastic pH to salinity stress. Short-term apoplastic alkalization in the root under salinity stress has been reported in *Arabidopsis* (Gao et al., 2004), although that study did not clarify whether the apoplastic alkalization occurred in the root elongation zone or the mature zone.

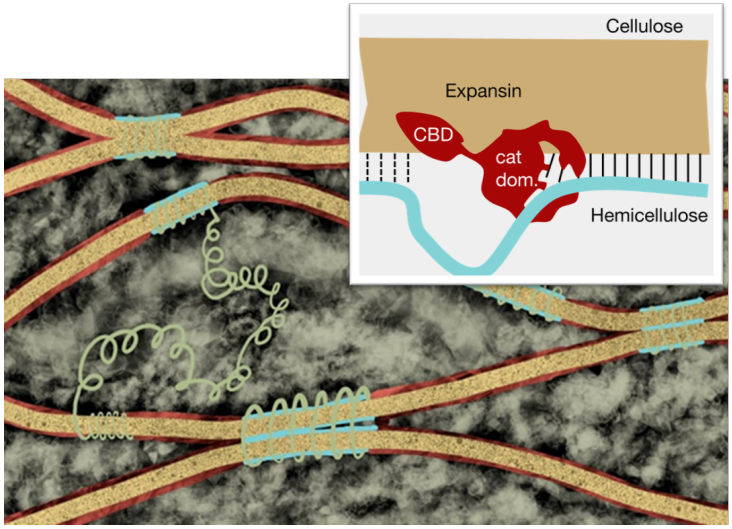

Figure 1-5. Schematic diagram of cell wall loosening by expansins. The hydrophobic region (blue) of cellulose microfibrils (yellow areas) are bound to various glycans such as xyloglucan or xylan (green coils). Expansin can bind to the hydrophobic region and unzip the non-covalent cross-links (black line) between cellulose and xyloglucan, resulting in a type of polymer creep. This schematic is cited from Cosgrove (2018).

1.6 Objectives

Wheat production has been severely affecting by soil salinization. It has been an urgent issue to improve wheat productivity in salinized soils. Therefore, it is essential to study the salinity tolerance mechanisms in wheat. To elucidate the functions of root cell wall in root growth under salinity stress, this study was conducted with the following objectives:

1) to investigate the chemical compositions and properties of root cell wall in relation with root growth under salinity stress.
2) to study the effect of extensibility of root cell wall on root growth under salinity stress.
3) to clarify the function of expansins in root extension under salinity stress.

Chapter 2. Chemical compositions and properties of root cell wall in relation with root growth under salinity stress

2.1 Abstract

Root cell wall composition has been associated with salt tolerance in plants. the cell wall composition of root tips in wheat (*Triticum aestivum* L.) cultivars and their related mechanisms of salt tolerance were evaluated. Two salt-sensitive (Yonliang-15 and GS-6058) and salt-tolerant wheat (JS-7 and Xinchun-31) cultivars were used. Salt stress decreased the pectin content in the first zone in all cultivars except JS-7. Hemicellulose I and II increased significantly in the first and second zones in sensitive cultivars compared to tolerant cultivars. Cellulose content increased significantly in all cultivars in both zones; this increment was more pronounced in sensitive cultivars than in tolerant cultivars. The uronic acid content in pectin in the first zone was significantly lower in sensitive cultivars than in tolerant cultivars, while the uronic acid content in hemicellulose showed an opposite tendency. The cation exchange capacity of the root cell wall was significantly lower in sensitive cultivars than in tolerant cultivars. A positive relationship existed between root growth, relative content of pectin in the total cell wall of the first zone, and cation exchange capacity of the root cell wall; however, root growth and the relative content of cellulose in the total cell wall were negatively associated. These results suggested that high pectin content and cation exchange capacity, as well as low hemicellulose and cellulose contents in the cell wall of wheat root might be useful for maintaining root growth and tolerance under salt stress conditions.

2.2 Introduction

Salinity is one of the major factors that affects crop growth, leading to severe damage and loss of yield (Zhu 2001). Plant growth largely depends on cellular growth and plant cell walls. Salt stress can indirectly affect the cell wall composition by causing changes in cell wall metabolism. For example, studies have shown that pectin modification (Yan et al. 2018), cellulose synthesis (Zhang et al. 2016), and hemicellulose synthesis (Li et al. 2013) are involved in response to salt stress in *Arabidopsis*. These results indicate that changes in cell wall composition are indispensable for plants to cope with salt stress. Particularly in the root tips, the cell wall composition is involved in cell elongation, thereby affecting root growth.

The cell wall is composed of relatively stiff cellulose microfibrils that are embedded in a hydrated matrix of pectin and hemicellulose (Cosgrove 2018). Cellulose and xyloglucan are the main load-bearing components of the cell wall (Cosgrove 2018), while pectin is associated with ion homeostasis (Koyama et al. 2001) and cell wall expansion (Palin and Geitmann 2012). In excised pea roots (*Pisum sativum*), salt increased uronic acid and cellulose contents (Solomon et al. 1987); in cotton (*Gossypium hirsutum*), salt decreased the cellulose and the total uronic acid contents of the cell wall in the elongation zone (Zhong and Lauchli 1993). Furthermore, in wheat roots, salt stress significantly decreased the pectin and cellulose contents but increased the hemicellulose content (Al-Hakimi and Hamada 2001); in soya bean (*Glycine max*), a salt-sensitive cultivar increased hemicellulose and cellulose contents and decreased pectin content in the root elongation zone under salt stress (An et al. 2014).

When the cells stop expanding in the differentiation zone, secondary cell walls are produced; in the secondary cell wall, hemicellulose, and pectin are replaced by cellulose microfibrils (Mellerowicz and Sundberg 2008). Notable differences in cell wall composition between the root elongation zone and the differentiation zone show the different strategies adopted by plants to cope with salt stress (Byrt et al. 2018). Further, in the elongation zone, the pectin cross-links

help to maintain the integrity of cell walls (Feng et al. 2018) and ion homeostasis under salt stress (Fang et al. 2019). Moreover, in the differentiation zone, the increased uronic acid content in the cell wall traps Na^+ and restrict its movement.

Cation exchange capacity (CEC) of the cell wall indicates the ability of cell walls to hold exchangeable cations, reflecting the possibility of cation interaction in cell walls. Salt tolerance in plants is associated with the CEC of cell walls. The roots of *Spinacia oleracea* (glycophyte) and *Suaeda altissima* (halophyte) have been reported to exhibit an increase in uronic acid content and CEC of cell wall in response to salt stress; these increments were more pronounced in the halophyte (Meychik et al. 2006). In chickpea (*Cicer arietinum*), salt stress increased the uronic acid content in two cultivars, and salt-tolerant cultivars showed high CEC in the isolated cell walls (Meychik et al. 2010). In barley (*Hordeum vulgare*), salt-tolerant cultivars showed greater Na^+ exchange capacity relative to salt-sensitive cultivars (Flowers and Hajibagheri 2001). In celeriac (*Apium graveolens*) and parsnip (*Pastinaca sativa*), pectin largely contributed to the CEC of the root cell wall (Szatanik-Kloc et al. 2017). Studies have also shown that hemicellulose interacts with cations and retains cadmium and aluminum in *Arabidopsis* (Yang et al. 2011; Zhu et al. 2013). This suggests that under salt stress, hemicellulose may also contribute to the cation exchange capacity of the root cell wall in the differentiation zone.

In wheat, salt tolerance related to osmotic (Cuin et al. 2009) and specific ion toxicity (Asgari et al. 2012; Jixiang Lin 2012) in cells have been extensively studied, however, the changes in root cell wall that are related to root growth and CEC under salt stress remain unclear. Therefore, the objectives of this study were to investigate the changes in root cell wall compositions of the root first (meristem and elongation zone) and second zone (differentiation zone), and assess the ability of cation exchange in cell walls of four wheat cultivars with

contrasting salt tolerance under salt stress conditions. Understanding these issues would be beneficial for further improving salt resistance in wheat.

2.3 Materials and methods

2.3.1 Materials

Among 10 different wheat cultivars, four spring wheat cultivars: Yongliang-15 (YL-15), GS-6058, JS-7, and Xinchun-31 (XC-31) were investigated. XC-31 was obtained from Xinjiang Agricultural University, while GS-6058, JS-7, and YL-15 were obtained from the Northwest Institute of Eco-Environment and Resource in Gansu, China. According to the previous review (Mujeeb-Kazi et al. 2019), root growth rate under salinity stress is closely related to salt tolerance in wheat. JS-7 and XC-31 were chosen as salt-tolerant cultivars, while YL-15 and GS-6058 as salt-sensitive cultivars following observations at the young seedling stage under 80 mM NaCl condition.

Grain's surface was sterilized in 0.5% sodium hypochlorite for 5 min, and rinsed three times with distilled water. Twenty grains of a cultivar were set on a sheet of filter paper and placed in a plastic bag, then, the plastic bag containing wheat grains was placed in a dark-conditioned growth chamber (SANYO MLR-350 HT, Japan) set at 25 °C. When roots were approximately 1.5 cm, they were exposed to NaCl solutions (0, 40, 80, and 120 mM, respectively) in 11 days. Each treatment contained 20 sheets. The growth chamber was then set at 60% relative humidity, 2000 lx for 16 h at 23 °C and 8 h at 18 °C in the dark. Root lengths were measured with calipers every day after NaCl treatments. Relative root growth under the 80 mM NaCl condition was calculated based on a root length of 100% for seedlings of each cultivar grown under 0 mM NaCl solution.

2.3.2 Composition of root cell wall

The roots were washed with distilled water. The segments of 0–5 mm (first zone: meristem and elongation zone) and 5–10 mm (second zone: differentiation zone) were excised and collected. Fresh weights of each replicate (approximately 160 segments) were recorded. The composition of the cell wall was determined according to An et al. (2014) with slight modification. Pectin, hemicellulose, and cellulose fractions of the cell wall were extracted by CDTA (trans-1,2-Diaminocyclohexane-N,N,N',N'-tetraacetic acid), KOH (potassium hydroxide), and sulfuric acid, respectively. The amounts of total sugar and uronic acid in each fraction were measured using the phenol-sulfuric acid method (Dubois et al. 1951) and meta-hydroxy diphenyl method (Blumenkrantz and Asboe-Hansen 1973), respectively.

2.3.3 Cation exchange capacity (CEC)

The CEC of the entire root cell wall was measured using the methods of Crooke (1964) and Meychik and Yermakov (2000) with slight modification. Excised roots were kept in an oven at 100 °C for 20 min and then dried at 50 °C for two days. Dried roots of each sample were placed in tubes and were further incubated and centrifuged in the following sequence: 10 mM KOH (three times), distilled water (once), and 10 mM HCl (3 times); these solutions were prepared with deionized water. The samples were finally washed with deionized water until the electrical conductivity of the supernatant dropped to 0. The root samples were then dried to a constant weight at 50 °C.

Root samples (0.04 g) of each replicate were placed in 15 mL tubes and 12.5 mL of NaCl solution (0, 10, 40, 80 mM) was added to each replicate. To reach a full ionic equilibrium, the samples underwent several agitations while they remained in the solution for 48 hours. The pH of the solution before and after the equilibrium was determined using a pH meter (HORIBA D24, Japan). The CEC of the cell walls was calculated with the following formula (1):

$$CEC = \frac{\left(10^{-pH^{eq}} - 10^{-pH^{in}}\right) \times V \times 1000}{g} \quad (1)$$

where CEC = cation exchange capacity of cell walls (mmol g^{-1} dry weight), pH^{in} and pH^{eq} = initial and equilibrium pH of the solution, V = volume of the solution (ml), and g = dry weight of the sample (g). Each treatment had three replicates.

2.3.4 Statistical analysis

Statistical analysis was performed using SPSS 21 software (IBM Corp., USA). The data were subjected to an analysis of variance (ANOVA), and the means were compared using Duncan's multiple range test ($P < 0.05$). Pearson correlations were used to determine the relationship between root growth, cell wall composition in the root first and second zones, and the CEC of the entire root cell wall.

2.4 Results

2.4.1 Root growth

The 80 mM NaCl solution dramatically inhibited root elongation in all cultivars, the root lengths of JS-7 and XC-31 were greater than those of YL-15 and GS-6058 under both 0 and 80 mM NaCl treatments (Fig. 2-1A). The relative root lengths of JS-7 and XC-31 were significantly higher than those of YL-15 and GS-6058 under the 80 mM NaCl treatment 11 days after germination (Fig. 2-1B). With regard to root growth, JS-7 and XC-31 were designated as tolerant, whereas YL-15 and GS-6058 were designated as sensitive cultivars.

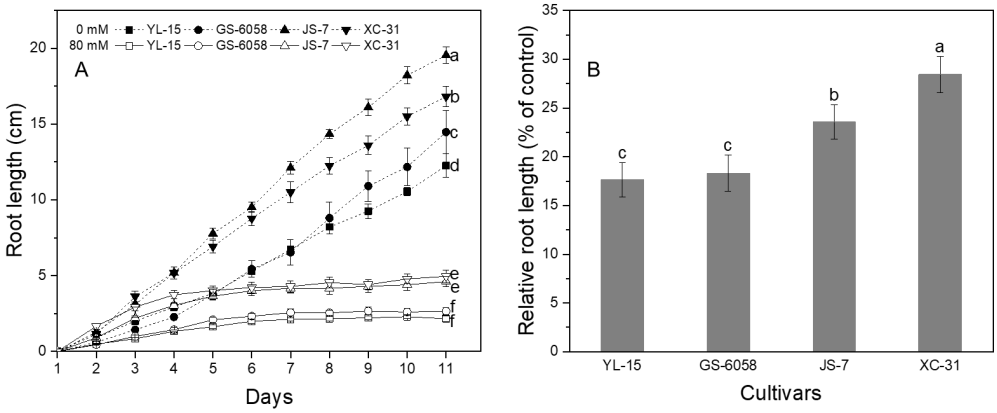

Figure 2-1 Root growth of four wheat cultivars under 0 mM and 80 mM NaCl treatments.

1) Root length of two salt-sensitive wheat cultivars (YL-15, square; GS-6058, round) and two salt-tolerant wheat cultivars (JS-7, triangle; XC-31, inverted triangle) under 0 mM (dash line) and 80 mM NaCl (straight line) treatments. Data are expressed as means ± S.E. (n = 20). Data followed by different letters indicate significant differences at $P < 0.05$.
2) Relative root length of two salt-sensitive wheat cultivars (YL-15 and GS-6058) and two salt-tolerant wheat cultivars (JS-7 and XC-31) under 80 mM NaCl treatments at 11 days after germination. The relative root growth was calculated based on a root length of 100% for plants of each cultivar grown without NaCl. Data are means ± S.E. (n = 20). Data followed by different letters indicate significant differences at $P <$ 0.05.

2.4.2 Composition of root cell wall

The data for root cell wall compositions were similar between 0 and 40 mM NaCl treatments, and between 80 and 120 mM NaCl treatments, therefore, only the data of 0 and 80 mM NaCl treatments are shown in Fig. 2-2. In the first zone, all cultivars under the 80 mM NaCl treatment except the JS-7 showed a decrease in the pectin content, moreover, there was no difference in pectin content in the second zone (Fig. 2-2). Concerning hemicellulose I (HC1) and hemicellulose II (HC2), under the 80 mM NaCl treatment, significant increases in both the first and second zones were observed only in the sensitive cultivars; except for HC1 in JS-7 in the first zone. The HC1 and HC2 in the second zone of tolerant cultivars remained similar with the 80 mM NaCl treatment. The cellulose content increased dramatically in both the first and second zones in all cultivars under the 80 mM NaCl treatments.

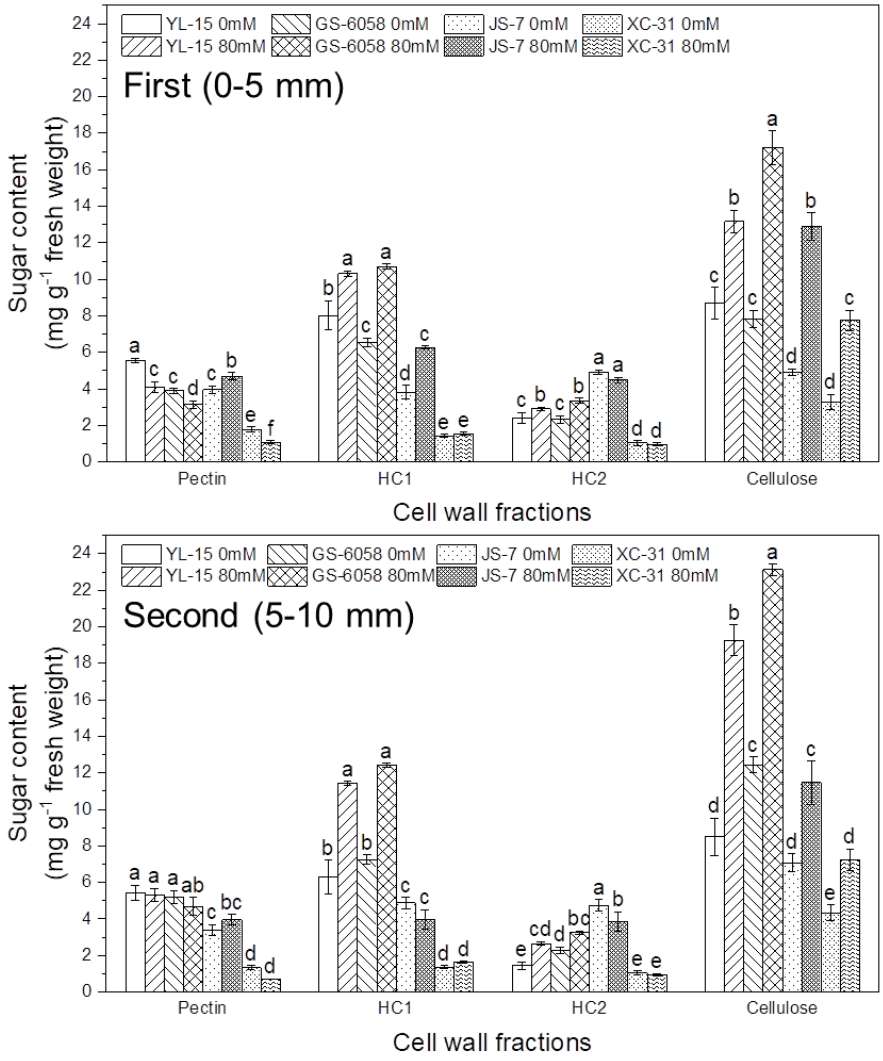

Figure 2-2 Total sugar contents of pectin, hemicellulose I (HC1), hemicellulose II (HC2), cellulose in elongation (0–5 mm) and second zones (5–10 mm) of root tips in salt-sensitive (YL-15, GS-6058) and salt-tolerant wheat cultivars (JS-7, XC-31) under 0 and 80 mM NaCl treatments. Data are expressed as means ± S.E. (n = 5). Data followed by different letters indicate significant differences at $P < 0.05$.

2.4.3 Uronic acid content in pectin, hemicellulose, and cellulose

In the first zone, the uronic acid content in pectin was significantly lower in the sensitive cultivars than in the tolerant cultivars with 80 mM NaCl treatment (Fig. 2-3). However, hemicellulose (HC1 and HC2) fractions showed an opposite tendency in both the elongation and second zones. The uronic acid content in cellulose increased dramatically in the first and second zones in all cultivars with NaCl treatments, except XC-31 in the first zone. The

magnitudes of increase in cellulose content were pronounced in the second zone of the salt-sensitive cultivars, which increased more than two times under the 80 mM NaCl condition.

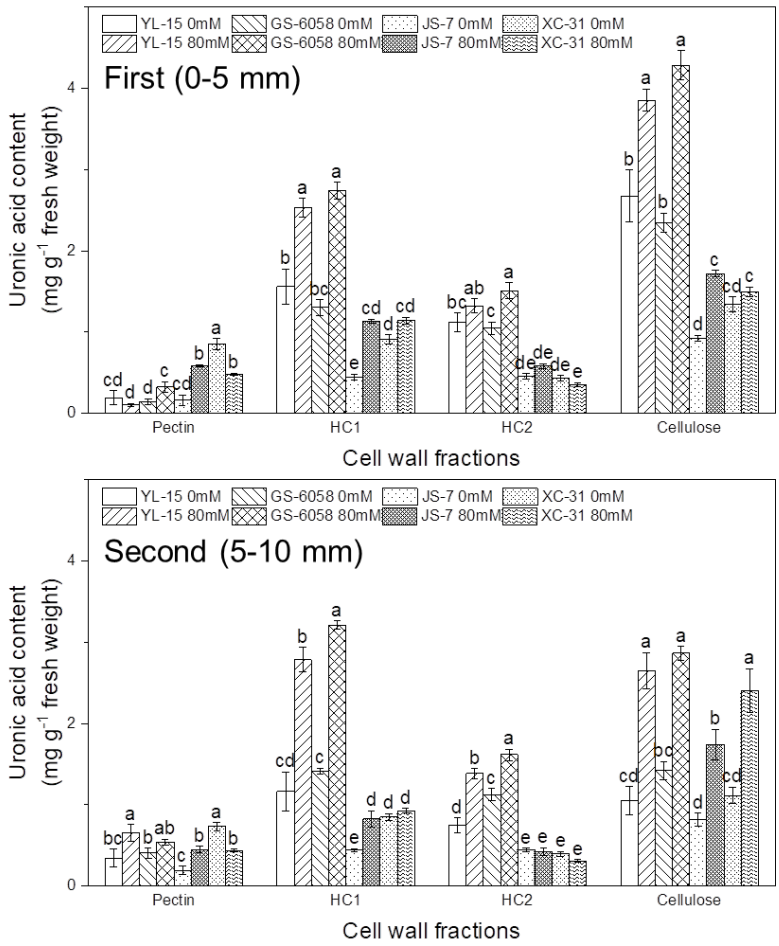

Figure 2-3 Uronic acid contents in pectin, hemicellulose I (HC1), hemicellulose II (HC2), and cellulose in the elongation (0–5 mm) and second zones (5–10 mm) of root tips in two salt-sensitive wheat cultivars (YL-15, GS-6058) and two salt-tolerant wheat cultivars (JS-7, XC-31) under 0 and 80 NaCl treatments. Data are expressed as means ± S.E (n = 5). Data followed by different letters indicate significant differences at $P < 0.05$.

2.4.4 Cation exchange capacity of root cell walls

To study the changes in CEC of the wheat roots, the CEC of the root cell walls at the solution ionic strength of 10, 20, 40, and 80 mM were measured. The CEC of the entire root of the four cultivars grown in 0 mM and 80 mM for 11 days are shown in Fig. 2-4. The CEC of the root cell walls in the four wheat cultivars treated with 0 and 80 mM showed the highest exchange capacity at the solution ionic strength of 80 mM. Salt treatment significantly decreased the CEC of the cell wall in all the wheat cultivars (Fig. 2-4). Therefore, the CEC of the root cell

walls in sensitive cultivars was significantly lower than the tolerant cultivars under the 0 mM and 80 mM treatments. The decrease of CEC was about 20% in all wheat cultivars except JS-7 under the 80 mM treatment (9.42%). Under 80 mM treatment, the salt-tolerant cultivars (XC-31 and JS-7) showed increase in CEC by 20.6% and 35.2%, respectively at the solution ionic strength of 80 mM as compared to solution of 10 mM, whereas the salt-sensitive cultivar showed less change in CEC.

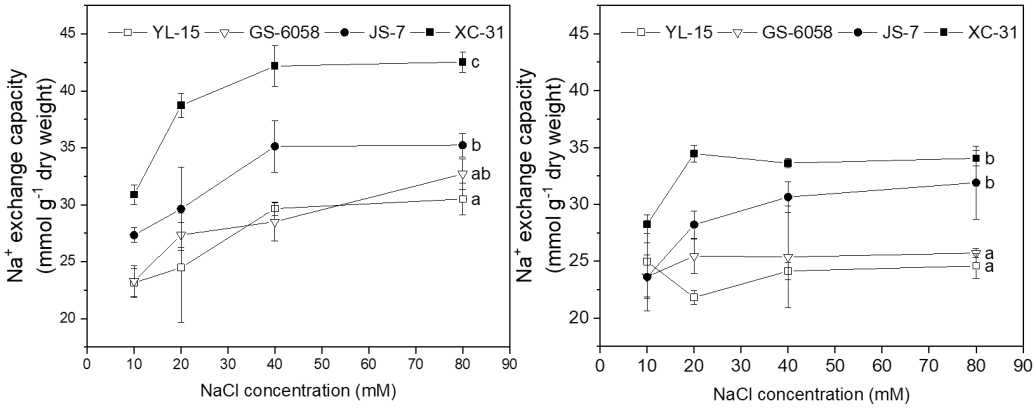

Figure 2-4 Cation exchange capacity of roots cell walls under 0 (left) and 80 (right) NaCl treatments was measured in a series concentration of NaCl solution (10, 20, 40, and 80 mM) in YL-15 (open square), GS-6058 (open triangle), JS-7 (solid round), and XC-31 (solid square). Data are expressed as means ± S.E. (n = 3). Data followed by different letters indicate significant differences at $P < 0.05$.

2.4.5 Correlations of cell wall related parameters

Salinity affected the cell wall composition in both zones. The first zone (0-5 mm) showed the initial response of root cell wall to the salinity stress. The second zone (5-10 mm) displayed further changes in the cell wall compositions under salt conditions, which may reflect the Na^+ sequestration ability of the cell wall in the root differentiation zone. The data of root growth, CEC and cell wall composition in both zones of JS-7 and YL-15 under 0 and 80 mM treatments were subjected to the correlation analysis. In addition, data regarding the first and second zones were subjected to correlation analysis (Table 2-1 and Table 2-2, respectively). The sugar and uronic acid content of each fraction in root tips (both first and second zone) were transformed into relative content because the relative sugar and uronic acid content of the cell wall fraction better reflected the effects of saline stress in different wheat cultivars. In the present study, the

root growth was positively related to the relative content of the pectin fraction (r = 0.87), and negatively related to the relative content of the cellulose fraction (r = -0.66) in the first zone (Table 2-1). The total cell wall content in the first zone showed a negative effect on the root growth (r = -0.68). While the CEC of the root cell walls showed a positive effect on root growth (r = 0.72). And the CEC of the root cell wall was positively related to the relative uronic acid content of the pectin fraction (r = 0.81), and negatively related to the total cell wall and total uronic acid (r = -0.91 and R = -.714, respectively), in the first zone (Table 1-1). Similarly, in the second zone, the CEC of the root cell wall was positively related to the relative uronic acid content of pectin (r = 0.76) and HC1 (r = 0.78) and negatively related to the total cell wall (r = -0.89) (Table 2-2).

Table 2-1 Correlation among the relative sugar content of pectin, hemicellulose I (HC1), hemicellulose II (HC2), and the cellulose fraction in the elongation zone (0–5 mm from the root cap), cation exchange capacity (CEC), total cell wall, and root length are shown in the table. The relative sugar contents were based on the percentage of individual fractions relative to that of the total cell wall.

		Relative uronic acid content (%)				Relative total sugar content (%)				Total uronic acid	Total cell wall	Root Growth
		Pectin	HC1	HC2	Cellulose	Pectin	HC1	HC2	Cellulose			
Relative uronic acid content	Pectin											
	HC1	.702										
	HC2	.380	.645									
	Cellulose	.496	.622	-.092								
Relative total sugar content	Pectin	.306	-.228	.228	-.458							
	HC1	-.619	.405	.405	-.742*	.011						
	HC2	-.039	-.476	-.476	-.377	.519	-.293					
	Cellulose	.275	-.110	-.110	.891**	-.773*	-.476	-.605				
Total uronic acid (mg g^{-1})		-.231	.131	.131	.191	-.832*	.347	-.750*	.590			
Total cell wall (mg g^{-1})		-.766*	-.149	-.149	-.591	-.399	.758*	-.175	-.188	.604		
Root Growth (mm)		.310	.225	.225	-.264	.869**	-.173	.624	-.660*	-.895**	-.682*	
CEC		.807*	.135	.135	.329	.609	-.701	.380	-.061	-.714*	-.912**	.722*

Data followed by * and ** indicate significant correlation at $P < 0.05$ and at $P < 0.01$, respectively.

Table 2-2 Correlation among the relative sugar content of pectin, hemicellulose I (HC1), hemicellulose II (HC2), and the cellulose fraction in the elongation zone (5–10 mm from the root cap), cation exchange capacity (CEC), total cell wall, and root length are shown in the table. The relative sugar contents were based on the percentage of individual fractions relative to that of the total cell wall.

		Relative uronic acid content (%)				Relative total sugar content (%)				Total uronic acid	Total cell wall	Root Growth
		Pectin	HC1	HC2	Cellulose	Pectin	HC1	HC2	Cellulose			
Relative uronic acid content	Pectin											
	HC1	.887**										
	HC2	.548	.172									
	Cellulose	.616	.829*	.105								
Relative total sugar content	Pectin	-.133	.136	-.525	-.011							
	HC1	.490	.487	-.097	-.016	.570						
	HC2	-.318	-.142	-.406	.114	.036	-.353					
	Cellulose	-.006	-.248	.578	-.048	-.840**	-.631	-.399				
Total uronic acid (mg g^{-1})		-.034	-.091	.187	.276	-.509	-.537	.347	.345			
Total cell wall (mg g^{-1})		-.671	-.836**	.001	-.589	-.324	-.533	.208	.328	.584		
Root Growth (mm)		.260	.180	.185	-.231	.475	.651	-.585	-.243	-.879**	-.538	
CEC		.760*	.782*	.200	.388	.411	.771*	-.312	-.434	-.606	-.892**	.722*

Data followed by * and ** indicate significant correlation at $P < 0.05$ and at $P < 0.01$, respectively.

2.5 Discussion

Root growth maintenance is an important trait for wheat growth and it is highly associated with salt tolerance (Mujeeb-Kazi et al. 2019). Under salt stress, the high relative root length is closely related to salt tolerance in wheat at the early seedling stage (Sadat Noori and McNeilly 2000). In this study, the JS-7 and XC-31 showed a 5%–10% increase in the relative root length as compared to YL-15 and GS-6058 (Fig. 2-1). Therefore, JS-7 and XC-31 were shown to be more tolerant to salt stress than YL-15 and GS-6058.

2.5.1 Cation exchange capacity in relation with salt tolerance

Wheat evaporates water 50 times more than it retains, which makes the shoots more sensitive to NaCl (Munns et al. 2020). To avoid the accumulation of Na^+ in shoots, wheat roots prevent more than 95% NaCl from entering the cells by extruding the excess Na^+ from the cytosol (Munns et al. 2020). Being the outmost layer of the cells, root cell walls are involved in constraining the movement of Na^+ to the stele, and ultimately to the shoots (Byrt et al. 2018). Furthermore, cell walls are negatively charged and they bind cations reversibly. Previous studies showed that in *Suaeda altissima* (Meychik et al. 2006) and barley (Flowers and Hajibagheri 2001), the CEC of the cell walls in the mature zone is highly associated with salt

tolerance. In this study, however, the decreased CEC under saline condition suggests that Na^+ affects the cell wall components that are involved in cation binding (Fig. 2-3 and Fig. 2-4). The high CEC under the saline condition and the increase in Na^+ concentration in the tolerant cultivars (Fig. 2-4) imply that the tolerant cultivars bind more Na^+ in the root cell wall than the sensitive cultivars, which may result to a reduced concentration of toxic Na^+ entering into the cells and transporting to the shoot. The high correlation among the CEC and root growth indicates that the chemical property of the root cell wall that is related to the CEC is an important factor that affects root growth under saline conditions.

2.5.2 Uronic acid in relation with cation exchange capacity

The uronic acid-rich polysaccharides are the main ion-binding sites in the cell walls under physiological conditions (Meychik and Yermakov 2000). It has been reported that uronic acid content in pectin contributes about half of the CEC in the root cell wall (Szatanik-Kloc et al. 2017). Previous studies showed that HC I retains cadmium and aluminum in *Arabidopsis* (Yang et al. 2011; Zhu et al. 2013), indicating that the ion-bind sites of hemicellulose could also interact with cations. In our study, the CEC of the total cell wall was positively correlated with the relative uronic acid content in pectin in the root first zone (Table 2-1), indicating that the uronic acid in pectin contributes to the interaction between Na^+ and the cell wall. In the second zone, the close relationship between the CEC of the root cell walls, relative uronic acid content in pectin, and HC1 (Table 2-2), indicates that the development of cell wall, the ion-binding sites of both pectin, and HC I may be involved in the enhancement of the CEC in the cell wall.

2.5.3 Root growth in response to salinity

The impact of abiotic stress on wheat cell wall polysaccharides has been studied previously (Al-Hakimi and Hamada 2001; Leucci et al. 2008). However, the changes in the composition

of cell wall polysaccharides in wheat root tips with differing salt tolerance under salt stress have not been reported. In the present study, the total sugar content of the cell wall was significantly increased in all wheat cultivars under salt stress after 11 days of germination. In contrast, previous studies showed a decrease in soybean under a 2-day salt stress (An et al. 2014). The discrepancies between our study and previous studies show the spatial-temporal changes of cell wall compositions, which may be related to the root growth under salt stress. The growth rate of the roots during the salt stress showed a period of quiescence (8 to 48 hours) and growth recovery thereafter (van Zelm et al. 2020). Salt-tolerant cultivars showed less decrease in the cell wall content than the salt-sensitive cultivars in soya bean after 40 hours of salt treatments, indicating that the maintenance of root cell wall components is important for the recovery of root growth under salt stress (An et al. 2014). Under the long-term salinity stress, the Na^+ decreased the cell wall-bound hydrolases, resulting in the deposition of cell wall and stunted growth in the roots (Singh and Prasad 2009). The same situation may have occurred in the present study; the negative correlation between the total cell wall content in the root first zone and root growth (Table 2-1) suggests that the increase in the total cell wall component by salt stress in all wheat cultivars might be associated with the inhibitory effects of salt on root growth. In addition, a lesser increment of the root cell wall components might contribute to the root growth under long-term salt stress in salt-tolerant cultivars (Fig. 2-2). This result also suggests salt stress may decrease cell division frequency and inhibit cell elongation, resulting in smaller cells and denser tissue. The thickness of cell wall and size of cell will be further studied by microscope and fluorescent antibody.

Table 2-3 Relative sugar contents of pectin, hemicellulose I (HC1), hemicellulose II (HC2), and the cellulose fraction in the elongation and adjacent zone of root tips under 0 and 80 NaCl concentrations. The relative sugar contents were based on the percentage of individual fractions relative to the total cell wall. Data followed by different letters indicate significant differences at $P < 0.05$.

Cultivars	Salt treatment	Pectin (% total cell wall)		HC1 (% total cell wall)		HC2 (% total cell wall)		Cellulose (% total cell wall)	
		Elongation	Adjacent	Elongation	Adjacent	Elongation	Adjacent	Elongation	Adjacent
YL-15	0 mM	22.56 efg	25.73 g	32.53 gh	29.08 ef	9.72 cd	6.67 a	35.19 b	39.14 bc
	80 mM	13.43 bc	13.72 bc	33.81 h	29.57 efg	9.55 cd	6.88 a	43.21 cd	49.83 ef
GS-6058	0 mM	18.87 de	19.06 de	31.81 gh	26.68 de	11.33 d	8.42 abc	37.99 bc	45.79 de
	80 mM	9.13 ab	10.75 ab	31.09 fgh	28.61 ef	9.77 cd	7.48 ab	50.00 ef	53.16 f
JS-7	0 mM	20.98 def	16.95 cd	26.85 de	24.27 d	26.13 h	23.61 g	26.04 a	35.20 b
	80 mM	17.60 cd	16.97 cd	17.30 bc	17.08 bc	16.83 f	16.62 f	48.27 def	49.33 ef
XC-31	0 mM	23.61 fg	16.51 cd	18.95 c	16.93 bc	13.80 e	13.05 e	43.64 cd	53.51 f
	80 mM	9.45 ab	6.79 a	13.73 a	15.44 ab	8.43 abc	9.07 bc	68.39 g	68.71 g

2.5.4 Cell wall composition in response to salinity

Salt stress significantly increased the cell wall content and altered the cell wall composition in the root first zone. With 80 mM salt treatment, pectin accounted for 17% of total cell wall composition in the root first zone in JS-7, while pectin accounted for 13% and 9% in YL-15 and GS-6058, respectively (Table 2-3). The relative cellulose content increased in the root elongation and second zones in all wheat cultivars under salt stress (Table 2-3). The cellulose and total cell wall contents were higher in the sensitive cultivars than in the tolerant cultivars with 80 mM salt treatment (Fig. 2-2). The correlation analysis showed that the root growth was positively related to the relative content of the pectin fraction in the first zone and negatively related to the relative content of cellulose fraction and the total cell wall in the first zone. This indicates that the high pectin content, low cellulose, and less accumulation of cell walls may contribute to the high root growth rate in salt-tolerant cultivars under salt stress.

Pectin encompasses a range of galacturonic acid-rich polysaccharides, the uronic acid-rich polysaccharides form a hydrogel matrix through cross-linking that is mediated by cations (O'Neill et al. 2004). The reduction of pectin cross-linking caused by Na^+ could reduce the

stabilizing influence of pectin in the cell wall (Feng et al. 2018). Mutant that has less cross-linking in pectin showed hypersensitive phenotypes to salt stress in *Arabidopsis* (Yan et al. 2018) and rice (*Oryza sativa*) (Fang et al. 2019). High amounts of uronic acid help to maintain the stabilization of cell wall and contributes to a hydrated environment around cell membranes, supporting a homeostatic microenvironment for cell wall bound hydrolases and ion transportation (Byrt et al. 2018). Consequently, in the present study, the high levels of uronic acid content (Fig. 2-3) might also contribute to root growth under salt stress in the salt-tolerant cultivars XC-31 and JS-7.

In the primary cell wall, the cellulose-hemicellulose-cellulose conjunctions are the main load-bearing components determining the elongation of cells (Cosgrove 2018). In the present study, HC I and II were significantly increased in both the elongation and second zones in the salt-sensitive cultivars as compared to the salt-tolerant cultivars under salt stress (Fig. 2-2). Under the 80 mM NaCl condition, the salt-sensitive cultivars showed higher HC I content and lower HC II content in the second zone than in the first zone (Fig. 2-2). Koyro (Koyro 1997) reported that salt stress altered the structure of cellulose microfibrils and hemicellulose, forming a stiffness-meshed network. The reduced growth rate in coffee (*Coffea arabica*) leaf cells under salt stress is mainly due to the increase in hemicellulos content, which forms stronger linkage between hemicellulose and cellulose (De Lima et al. 2014). The decrease in the content of hemicellulose in salt-tolerant cultivars (Fig. 2-2 and Table 2-3) may result in less conjunctions between hemicellulose and cellulose in the cell walls, alleviating the growth inhibition caused by the increased cellulose contents.

In conclusion, salinity stress decreased pectin and increased cellulose and total cell wall contents, which showed different patterns compared with the short-term alteration of cell wall composition in response to salinity. The CEC of cell walls and hemicellulose content in cell wall may play important roles on maintaining root growth and low Na^+ content in plants. The

present results also provide valuable insights of cell wall composition enhancing salt tolerance in wheat. However, the mechanism of how wheat root regulates the synthesis of hemicellulose and forms the linkage between the hemicellulose and cellulose under salinity stress needs to be further studied.

Chapter 3. Extension of root cell wall in relation with root growth under salinity stress

3.1 Abstract

Two salt-sensitive (Yongliang-15, GS-6058) and two salt-tolerant (JS-7, Xinchun-31) wheat cultivars were used to investigate the extension, extensibility (viscoelastic parameters), and chemical composition of the cell walls in their root elongation regions (apical 10 mm-long root segments), under salinity stress. After 10 days of saline (80 mM NaCl) or control (0 mM NaCl) treatments, elasticity of the root cell wall, indicated by E_0, significantly decreased in the salt-sensitive cultivars, whereas the E_0 in the salt-tolerant cultivars was maintained at the same level as that in the non-saline condition. Root extension and the differences among cultivars were largely dependent on elastic extension, which accounts for one-half to two-thirds of the total extension. Viscosity, indicated by η_0, and the plastic extension of the root cell walls did not change across the treatments and cultivars. The significant decrease in cell wall elasticity in the root elongation region was one of the factors that depressed root growth in salt-sensitive cultivars under the saline condition. The well-maintained elasticity of salt-tolerant cultivars alleviated the depression of root growth by NaCl. Cell wall elasticity was positively correlated with the relative pectin and hemicellulose I contents and negatively correlated with the relative cellulose content. Under saline conditions, the relative hemicellulose II content did not change in the salt-sensitive cultivars; however, it decreased in the salt-tolerant ones. Thus, changes in chemical composition of the cell wall correspond with the cell wall extensibility and root growth in wheat cultivars with different degrees of salt tolerance.

3.2 Introduction

Root growth is vital for the whole plant growth under saline conditions (An et al. 2002), and depends on cell division and elongation in the elongation zone. Driven by turgor pressure, the cells keep expanding while the cell walls keep extending until they become too rigid to extend (Cosgrove 2018). Salinity decreases the turgor pressure due to osmotic stress (Rygol and Zimmermann 1990; Ogawa and Yamauchi 2006). Therefore, wall extensibility is one of the most important factors for root elongation under saline conditions.

Cell wall extension, composition, structure, and growth dynamics have been extensively reviewed by Cosgrove (2018). Cell walls are composed of stiff cellulose microfibrils embedded in a hydrated matrix of pectin and hemicellulose. Pectin governs the cell wall extensibility by affecting the cell wall elasticity (Wolf and Greiner 2012). Recent studies have reported extensive pectin-cellulose interactions (Wang et al. 2015) and pectin-xylan links (Tan et al. 2013) in cell walls. Hemicellulose I is composed of long polysaccharide chains and is associated with cellulose microfibrils (Zhong and Lauchli 1993; Fry 2011). Hemicellulose II adheres to the surface of cellulose and forms cellulose-xyloglucan-cellulose conjunctions, which are major load bearing points for mechanical forces (Park and Cosgrove 2012; Zhang et al. 2014). Cellulose contributes to wall rigidity and mechanics (Zhang et al. 2014). The primary cell wall behaves like a viscoelastic composite material that demonstrates a time-dependent extension under load and time-dependent shrinkage after stretching (Boudaoud 2010; Cosgrove 2018). A Kelvin-Voigt-Burgers model with four elastic (E_0, E_1, E_2, E_3) and four plastic (η_0, η_1, η_2, η_3) parameters effectively analysed cell wall extension and shrinkage in the creep-extension analysis (Tanimoto et al. 2000). Cell wall extension is partially elastic and partially plastic (Boudaoud 2010), and the elastic and plastic parameters determine the elastic and plastic extension, respectively. Such extension (deformation) is a result of the polymeric

nature of the cell wall, however, the mechanical properties are largely unknown (Cosgrove 2018).

Changes in the cell wall composition in relation to the cell wall extensibility have been reported. Pectin and de-esterification of pectic homogalacturonan have been associated with wall stiffening and growth cessation (Siedlecka et al. 2008; Hongo et al. 2012; Wang et al. 2020). An increase in the phenolics and lignin in the cell wall, caused by water deficit, reportedly reduces the cell wall extension in the root elongation zone (Fan et al. 2006). Xyloglucan hydrolase decreases the amount and molecular mass of xyloglucans, which has been shown to increase the cell wall extensibility in the azuki bean (Kaku et al. 2002). The increase in the hemicellulose and ferulic acids in the cell wall, induced by Al, reportedly increases wall rigidity in wheat (Tabuchi and Matsumoto 2001). A decrease in the amount of xyloglucan increases the elastic and viscous extensibility of the apical root in tea (Safari et al. 2018). Hydrolysation of hemicellulose increases the wall extensibility in tomato hypocotyls (Miedes et al. 2011). In *Arabidopsis*, xyloglucan-deficient walls are more easily extended than normal walls (Xiao et al. 2016). A low concentration of bifunctional endoglucanases (which have the ability to cut both xyloglucan and cellulose) make the cell wall more deformable (Park and Cosgrove 2012). A denser assembly of cellulose microfibrils induces wall stiffness (Podgórska et al. 2017). A rice mutant with a defect in root elongation showed a significantly low extensibility and high cellulose and hemicellulose II contents in the root cell wall in the elongation zone (Inukai et al. 2012). Three classes of enzymes have been suggested to be involved in the cell wall elongation: EGases, XTHs, and glycosidases (Fry 2004). Collectively, these previous reports indicated that the chemical composition and extensibility of the cell wall inherently interact and sensitively respond to the growth environment.

Studies on the effects of abiotic stresses on the root cell wall extension are fairly limited. Water deficit has been shown to reduce the cell wall extensibility of the root elongation zone

in maize (Fan et al. 2006). Excessive aluminium (Al) in culture media depressed cell wall extension in the root apical zone in wheat (Tabuchi and Matsumoto 2001; Ma et al. 2004). Application of silicon (Si) increased the elastic extension and viscocity of the apical root cell wall in sorghum under drought conditions (Hattori et al. 2003). Compared to roots, the hypocotyl and leaves have been more extensively studied. Water deficit reportedly decreased the cell wall extensibility in the hypocotyl of soybean (Wu et al. 2005), and drought stress decreased the cell wall elasticity in rose leaves (Al-Yasi et al. 2020). Si application was shown to increase the leaf cell wall extensibility in rice, oat, and wheat seedlings (Hossain et al. 2002), and lead exposure reduced the leaf cell wall extensibility in rice (Hossain et al. 2015). NH_4^+-toxicity reportedly increases the cell wall rigidity, which limits the expansion of leaf cells (Podgórska et al. 2017). Auxin has recently been found to stimulate cell elongation by increasing the wall extensibility (Barbez et al. 2017; Majda and Robert 2018). Abiotic stresses seem to generally depress the cell wall extensibility; however, the effects of salinity (Na^+ ions) on root cell wall extension and extensibility have not yet been reported.

Under saline conditions, higher proportions of pectin and lower proportions of cellulose have been associated with cultivar differences in root growth in soybean (An et al. 2014a). The widely reported elevation effect of calcium (Ca) application on root growth under salinity stress was partially attributable to enhanced pectin levels (An et al. 2014b). A lower proportion of wall cellulose in the hypocotyls of squash and cultured tobacco cells ameliorated the inhibition in cell expansion and elongation under salinity stress (Sakurai et al. 1987; Iraki et al. 1989). The structural arrangement of cellulose microfibrils was altered by salt exposure in sorghum (Koyro 1997). The amount of cellulose in the primary root was shown to decrease in response to salinity stress in cotton (Zhong and Lauchli 1993) and soybean (An et al. 2014a). In *Artemisia annua*, the main changes in the cell wall were found in the structure of pectin under salt stress (Corrêa-Ferreira et al. 2019). Feng et al. (2018) reported that salinity damaged the

cell walls in *Arabidopsis* by disrupting pectin crosslinking. Wang et al. (2020) reported that sodium induced pectin de-esterification, which reduced cell wall stiffness in isolated onion epidermel cells. The extension coefficient of wheat leaves was decreased even under short-term salinity exposure (Veselov et al. 2009). While the genes encoding xyloglucan-related enzymes, which are functional in the enhancement of root growth, were upregulated under long-term salinity exposure (Mahajan et al. 2020).

However, cultivar differences in root growth in relation to the cell wall extensibility, extension, and compositions in crops have not been reported previously. Therefore, the present study investigated the root cell wall extension parameters and extension and chemical compositions in the elongation region of young wheat seedlings under saline and non-saline conditions. The aim of the study was to understand the interactions among the extensibility and extension of the root cell wall, chemical composition, and root growth in wheat under saline conditions.

3.3 Materials and methods

3.3.1 Cultivation of wheat seedlings

Based on the growth and yield of the cultivars grown in saline soils in the northwest of China (personal communication with local researchers), two salt-sensitive (Yongliang-15, GS-6058) and two salt-tolerant (JS-7, Xinchun-31) wheat cultivars were selected as the experimental materials. Seeds of the four cultivars were surface sterilised in 5% sodium hypochlorite (NaOCl) for 5 min and then rinsed with distilled water three times. Twenty seeds were placed in a line on a sheet of filter paper. Each prepared sheet of filter paper (with the wheat seeds) was placed in a 24 × 34 cm plastic zipper bag and moistened with distilled water. The plastic bags containing the seeds were vertically placed in growth chambers (SANYO MLR-350 HT, Japan) set at 25 °C. Two days later, when the roots and leaves had reached lengths of ~1.5 cm and 1 cm, respectively, 80 mM NaCl (saline treatment) solutions with 1/12 fold of Hoagland solution

were reinsed on roots everyday. The same solution without NaCl was used as the control (non-saline treatment). Excess solutions were drained. During the treatment period in the growth chambers, plants were exposed to light (2000 lx) conditions of 16/8 h (light/dark) and temperatures of 23/18 °C (day/night).

The lengths of all primary and seminal roots (usually three roots were generated from one seed) were measured daily. Ten days after the NaCl treatments, when there were significant differences in root length between the sensitive and tolerant cultivars in the 80 mM NaCl treatment group, roots of the seedlings were sampled for extension and chemical composition analysis.

3.3.2 Root cell wall extension

The extension of the apical root cell wall was determined following the method developed by Tanimoto et al. (1997, 2000). Apical root segments (10 mm-long) were excised and placed into boiled ethonol (80 °C) for 5 min to inactive the proteins. The segments were stored in ethonol at 4 °C. Before the extension measurement, the root segments were hydrated with distilled water at room temperature (about 20 °C) for 15 min. The diameter of the root at 5 mm behind the root tip, i.e. the middle part of the elongation zone, was measured under a microscope (Vitiny UM12, China) and the actual extension was determined using the computer program Creep meter (Yamaden RE2-3305C, Japan).

For each treatment, 30–50 roots from 4–5 growth bags were successfully measured. Sections of the root region between 3 to 8 mm behind the root tip (5 mm-long section) were subjected to the extension measurements. The root was secured between two clamps of the creep meter. A tensile force of 0.05 N was found to be optimal for obtaining typical clean and stable creep extension curves for these wheat roots, and tensile forces higher than this value resulted in some roots breaking soon after extension. Roots were extended for 5 min and then released for another 5 min. The extension was recorded by a computer at 0.2 second intervals. During the

measurement, roots were kept in a drop of distilled water to prevent drying out. Elastic parameters (E_0, E_1, E_2, E_3), the plastic parameter, the viscosity coefficient (η_0, η_1, η_2, η_3), and the total, elastic, and plastic extension distances were determined by the computer program, based on the Kelvin-Voigt-Burgers model (Tanimoto et al. 2000). Refer to Tanimoto et al. (2000) for detailed information regarding the root extension measurements.

Because the tensile force was fixed at 0.05 N for all roots, the extension distance would be shortened in the case of thick roots, even if they exhibited the same extensibility. To eliminate the effect of root thickness on extension distance, the values were converted as the extension distance under the 80 mM NaCl treatment proportionally to the increase in the area of the root cross sections. i.e. The converted extension = measured extension distance $\times(1+ (S_{80}-S_0)/S_0)$. Where S_{80} and S_0 are the areas of root cross section under 80 and 0 mM NaCl.

3.3.3 Chemical compositions in root cell wall

Roots were rinsed with distilled water three times, and then 10 mm-long apical segments were excised with a razor blade. Root samples from 4–5 bags containing ~160 root segments represented one replicate and 4–5 replicates were taken per treatment. The fresh weights of these segments were recorded. Some segments were assigned for dry weight measurement, i.e. placed in an oven set at 90 °C for 3 days prior to measurement. The water content of all cultivars under the control and salinity treatments were calculated. Based on the water content, the dry weights of the segments were calculated to determine the composition measurements. Cell wall compositions were analysed using the procedure of Zhong and Lauchli (1993) with minor modification. Specifically, root segments were homogenised with ice-cold Tris-HCl buffer (pH 7.4) and Tris buffer-saturated phenol using a μT-12 bead crusher (Taitec Corporation, Koshigaya, Japan). The homogenate was centrifuged with 15 minutes, 5 000 g at 10 °C. The supernatant was discarded and the pellet containing the cell walls was further purified by sequential incubation and centrifugation in cold Tris-HCl, ethanol, acetone, a mixture of

methanol: chloroform, and again acetone and ethanol. Cell wall extracts were treated with pronase in phosphate (pH 7.0). The walls were further treated with CDTA, 1 and 4 M KOH for pectin, hemicellulose I, and II extraction. Residual insoluble sediments were designated as the 'cellulose fraction'. The amount of total sugar in each fraction was measured using the phenol-sulphuric acid method (Dubois et al. 1951) and the meta-hydroxy diphenyl method (Blumenkrantz and Asboe-Hansen 1973).

3.3.4 Statistical analysis

All data were analysed using an ANOVA and the means were compared using Duncan's multiple range test at $P < 0.05$. Correlations among the compositions, extension distances, elastic parameters, and plastic parameters of the root cell wall and root growth were analysed by Pearson's correlations at $P < 0.05$. SPSS 21 software (IBM SPSS, USA) was used for all statistical analyses.

3.4 Results

3.4.1 Root growth

Salinity severely depressed root growth in all cultivars (Table 3-1). The relative root growth in the sensitive cultivars (Yongliang-15, GS-6058) was lower than that of the tolerant cultivars (JS-7, Xinchun-31). Compared with the control, the roots became thicker under NaCl treatment, i.e., root diameters increased by ~10% and 40%, and the area of the root cross sections increased by ~1.2 and ~1.9 times in the sensitive and tolerant cultivars, respectively.

Table 3-1 Root growth, diameter, and cross-sectional area of four wheat cultivars under 0 and 80 mM NaCl treatments

Cultivars	NaCl (mM)	Root length (cm)	Relative root growth (%)	Root diameter (mm)	Area of cross section (mm^2)	Increase in the cross-sectional area (%)
Yongliang-15	0	14.10 ± 0.58	100	0.524 ± 0.027	0.216 ± 0.014	0
	80	5.15 ± 0.08	37	0.590 ± 0.005	0.273 ± 0.005	23
GS-6058	0	13.29 ± 0.92	100	0.531 ± 0.002	0.223 ± 0.003	0
	80	3.77 ± 0.19	28	0.581 ± 0.005	0.264 ± 0.005	18
JS-7	0	13.80 ± 0.14	100	0.426 ± 0.003	0.143 ± 0.002	0
	80	6.59 ± 0.33	49	0.590 ± 0.023	0.274 ± 0.023	93
Xinchun-31	0	15.84 ± 0.27	100	0.484 ± 0.001	0.184 ± 0.003	0
	80	8.98 ± 0.13	57	0.637 ± 0.027	0.320 ± 0.028	78

3.4.2 Extension of root cell wall

Table 3-2 Distribution of elastic parameters and viscosity coefficients of root cell walls of four wheat cultivars under 0 and 80 mM NaCl treatments

Cultivars	NaCl (mM)	Elastic parameters				Viscosity coefficients			
		E_0 (10^6 Pa)	E_1 (10^7 Pa)	E_2 (10^7 Pa)	E_3 (10^7 Pa)	η_0 (10^{10} Pa s)	η_1 (10^9 Pa s)	η_2 (10^8 Pa s)	η_3 (10^7 Pa s)
Yongliang-15	0	1.65 ± 0.21c	3.12 ± 0.25ab	3.31 ± 0.93a	3.60 ± 0.20a	2.07 ± 0.25b	1.30 ± 0.09a	2.03 ± 0.07a	4.33 ± 0.42a
	80	3.33 ± 0.15ab	3.00 ± 0.25b	3.09 ± 0.47a	4.15 ± 0.39a	2.58 ± 0.26b	1.21 ± 0.12a	1.76 ± 0.23a	5.12 ± 0.71a
GS-6058	0	1.95 ± 0.14bc	3.73 ± 0.19ab	3.40 ± 0.92a	4.64 ± 0.05a	2.69 ± 0.30b	1.56 ± 0.12a	2.02 ± 0.08a	4.89 ± 0.37a
	80	3.64 ± 0.44a	3.49 ± 0.35ab	3.51 ± 0.13a	4.19 ± 0.40a	1.83 ± 0.21b	1.37 ± 0.14a	1.81 ± 0.08a	4.76 ± 0.44a
JS-7	0	3.71 ± 0.49a	3.82 ± 0.14ab	3.13 ± 0.18a	3.83 ± 0.38a	2.86 ± 0.47b	1.68 ± 0.04a	2.03 ± 0.08a	4.34 ± 0.35a
	80	3.37 ± 0.50ab	3.41 ± 0.40ab	2.86 ± 0.29a	3.59 ± 0.39a	2.53 ± 0.30b	1.43 ± 0.14a	1.66 ± 0.16a	4.23 ± 0.58a
Xinchun-31	0	3.40 ± 0.29ab	4.34 ± 0.69a	3.35 ± 0.81a	3.64 ± 0.92a	3.55 ± 0.65ab	1.72 ± 0.32a	1.85 ± 0.26a	4.52 ± 1.00a
	80	4.04 ± 0.91a	4.28 ± 0.51ab	3.98 ± 0.11a	4.89 ± 1.57a	5.31 ± 1.66a	1.60 ± 0.10a	1.79 ± 0.35a	3.75 ± 1.28a

Values represent means ± SEs (n = 17–51)
Means followed by the same letter in the same column are not significantly different ($P < 0.05$)

The results of the elastic parameters and viscosity coefficients are shown in Table 3-2, wherein an increase in the E_0 value indicates a decrease in elasticity. The E_0 values in the sensitive cultivars were significantly increased after the 80 mM NaCl treatment, i.e. to almost double the values observed under the control treatment, However, no significant changes were observed in the tolerant cultivars after NaCl treatment. Under the non-saline condition, the E_0 values of the sensitive cultivars were significantly lower compared with those of the tolerant cultivars. The elastic modules of E_1, E_2, and E_3 approximately ranged from 2.78×10^7 to 4.87×10^7 Pa in all treatments, i.e. were ~10 times higher than E_0. No significant differences were observed among the E_1, E_2, and E_3 modules between the 0 and 80 mM NaCl conditions for all cultivars, and no significant differences were detected in the viscosity modules (η_0, η_1, η_2, η_3) across cultivars and treatments.

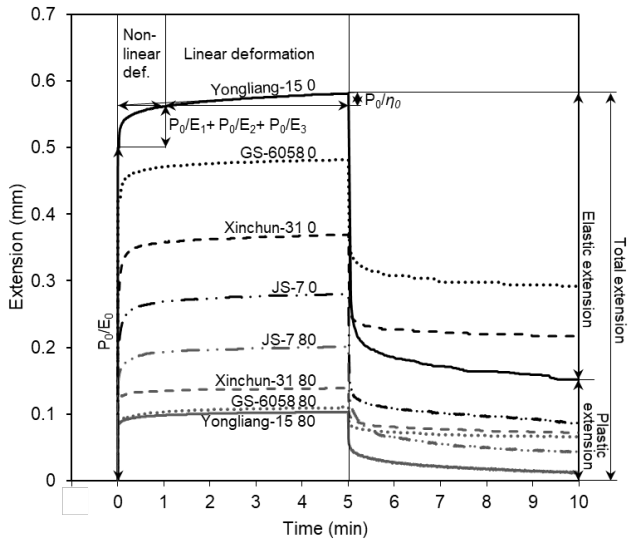

Fig. 3-1 Typical creep extension curves of root cell walls during the extension (5 min) and shrinkage (5 min) of four wheat cultivars under 0 (black lines) and 80 (grey lines) mM NaCl treatments. The total, elastic, and plastic extensions were determined by reading the extensions at 5 and 10 min. The schematic illustration is based on the extension curve of Yongliang-15.

P_0/E_0: linear instantaneous deformation

$P_0/E_1+ P_0/E_2+ P_0/E_3$: non-linear deformation

P_0/η_0: creep deformation

Typical root extension curves of all cultivars under both 0 and 80 mM NaCl treatments were successfully obtained using the setting conditions. Representative extension curves are shown in Fig. 3-1. As expected, salinity treatment depressed the root extension and this depression was much more prominent in the salt-sensitive cultivars than in the tolerant cultivars. Extension and viscoelastic parameters are simply illustrated in Fig. 3-1. Further details regarding the extension curves have been described by Tanimoto et al. (2000). The elastic, plastic, and total extensions are shown in Fig. 3-2. The directly measured total extension distances of roots were decreased by about 40–60% after 80 mM NaCl treatment in all cultivars (Fig. 3-2A). The elastic and plastic extension distances were generally decreased by the salinity treatment in all cultivars (Fig. 3-2A). The converted extension distances, which eliminated the effect of root thickness on the salinity treatment, were all increased in the four cultivars because the NaCl treatment caused the roots to thicken (Fig. 3-2B). However, a significant decrease in the elastic and total extension in the sensitive cultivars was still observed after treatment with 80 mM NaCl, compared with the control. While no significant differences were detected in the elastic,

plastic, and total extension results between the 0 and 80 mM NaCl treatments in the tolerant cultivars, elastic extension accounted for approximately one-half to two-thirds of the total extension in all cultivars and treatments, and plastic extension accounted for half or less than half of the total extension.

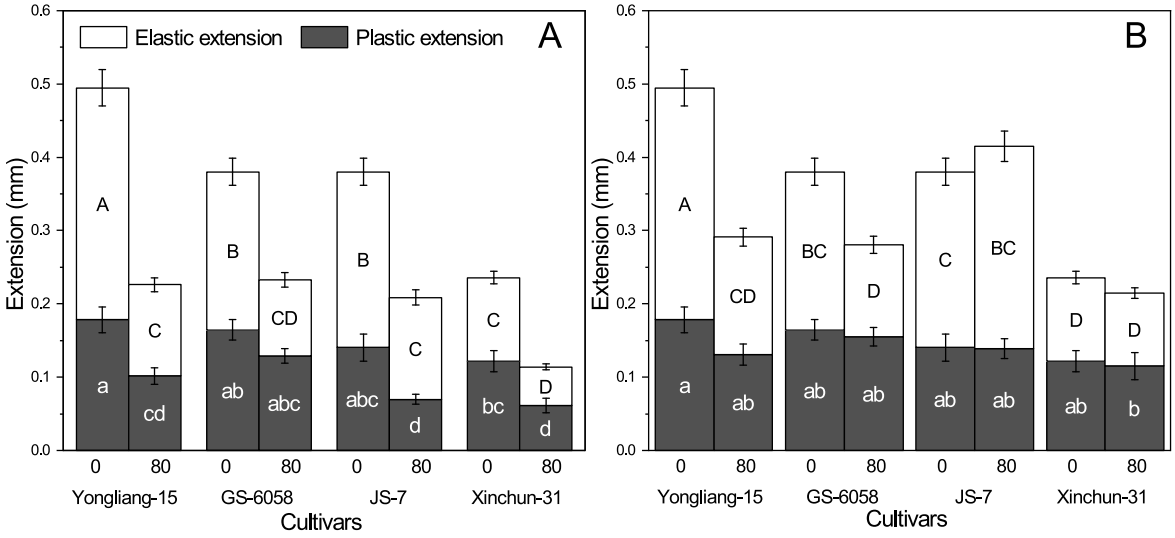

Fig. 3-2 The elastic, plastic, and total extensions of the root cell wall of four wheat cultivars under 0 and 80 mM NaCl treatments. A: Data were directly measured using a creep meter. B: Converted data that account for changes in root thickness. Data represent means ± SEs (n = 17–51). Different upper- and lowercase letters indicate significant differences ($P < 0.05$) in the elastic extension and plastic extension, respectively

3.4.3 Chemical composition of root cell wall

The chemical compositions and their relative amounts are shown in Fig. 3-3. The relative contents of the cell wall compositions were consistent with their absolute values. Irrespective of the wheat cultivars, no significant differences were detected in the total amounts of the root cell wall in the 10 mm-long apical root segments between the 0 and 80 mM NaCl treatments. However, the relative content of the four compositions (pectin, hemicellulose I, hemicellulose II, and cellulose) differed greatly in response to the NaCl treatment. The relative pectin content decreased, whereas the relative cellulose content increased in all cultivars under the saline condition. The tolerant cultivars showed significantly low relative hemicellulose I contents compared with the sensitive cultivars. The sensitive cultivars showed no significant changes in the relative hemicellulose contents, but the tolerant cultivars showed a significant decrease

under the saline condition. Notably, the total cell wall content in Xinchun-31 was only about half that of the other cultivars.

Correlations among the root extension parameters and the cell wall compositions are shown in Table 3-3. It is noteworthy that negative correlations were detected between E_0 and relative pectin, E_0 and relative hemicellulose I, relative pectin and relative cellulose, and relative hemicellulose I and η_0; and positive correlations were detected between the root growth, the total and plastic extensions, and the relative pectin contents. In addition, when using the calculated extensions, a positive correlation was observed between the root growth and elastic extension.

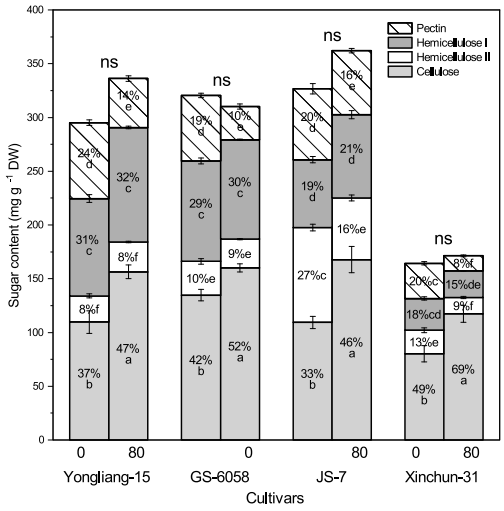

Fig. 3-3 Relative and absolute contents of pectin, hemicellulose I, hemicellulose II, and cellulose in the root cell wall of four wheat cultivars under 0 and 80 mM NaCl treatments. Values inside the bars indicate the relative values. Data represent means ± SEs (n = 5). Different letters within the same composition indicate significant differences in the relative content ($P < 0.05$). ns: no significant difference in the total cell wall content between 0 and 80 mM NaCl treatments within the same cultivar

3.5 Discussion

The tolerant cultivars (JS-7, Xinchun-31) showed higher relative root growth than the sensitive cultivars (Yongliang-15, GS-6058), which is consistent with their growth and production in real saline soils. Many previous reports have shown that wheat root growth is consistent with the whole plant growth under saline conditions (Sadat Noori and McNeilly 2000; Aslan et al. 2016; Mujeeb-Kazi et al. 2019). Therefore, the root length at the early

seedling stage can be used as a reliable salinity tolerance parameter for wheat cultivars. Hereafter, discussions regarding the salinity tolerance are based on the observed root growth.

3.5.1 Extension curve of root cell wall

Extensibility of the cell wall is an important factor that regulates cell elongation in plant tissue (Sakurai 1991; Cosgrove 2018). Extension curves of the root cell wall (subjected to measurement using a creep meter) have only previously been reported for green peas, i.e. the first attempt using a creep meter to obtain the extension curve of the root cell wall (Tanimoto et al. 2000). The extension curves of wheat roots under both saline and non-saline conditions in this study showed similar shapes to that reported for green peas (Fig. 3-1). This result confirmed that the mechanical properties of plant roots, even thin wheat roots, follow the Kelvin-Voigt-Burgers viscoelastic model (Tanimoto et al. 2000). In this model, modules E_0 and η_0 are the most significant parameters that indicate the elastic and viscous properties of the root cell wall, respectively (i.e. where higher E_0 values indicate lower extensibility). In the present study, the extension curves intuitively illustrated the cultivar differences in root cell wall extension and the effects of salinity on cell wall extension. The largely depressed extension in the sensitive cultivars indicated that the mechanical properties of the root cell wall of these cultivars were very sensitive to salinity stress.

3.5.2 Elastic parameters in relation with root growth

E_0 values have been reported for only three plants, i.e. green pea (Tanimoto et al. 2000), sorghum (Hattori et al. 2003), and Arabiadopsis (Shigeyama et al. 2016). In the present study, the E_0 of the elongation region of salt-sensitive cultivars under the non-saline condition ranged from 1.6 to 1.8 10^6 Pa (Table 3-2). These values were similar to those reported for green pea and sorghum roots (1.6–2.6 10^6 Pa) but were 10 times those reported for Arabiadopsis stems (1.8–3.2 10^5 Pa). The E_0 of the tolerant cultivars was higher than that of the above-mentioned plants, i.e. ~3.5 10^6 Pa. Salinity increased the E_0 in the sensitive cultivars but had no effect on

that in the tolerant cultivars (Table 3-2). These results are very similar to those seen for Al stress, e.g. Al increased the E_0 in Al-sensitive wheat cultivar but had no effect on the E_0 in the tolerant cultivar (Ma et al. 2004). This previous report suggested that Al binding with the cell wall resulted in the deformation of the cell wall, which increased the E_0 and reduced the exentsibility. Sodium (Na^+) also directly binds with cell walls, and, the ion-binding was reportedly much lower in tolerant cultivars compared with sensitive cultivars in barley and *Silene paradoxa* (Flowers and Hajibagheri 2001; Colzi et al. 2012). Therefore, the increased E_0 values in the sensitive cultivars may have been partially due to excesive Na-binding with the cell walls, although the Na binding in the tolerant cultivars may have been insufficient to cause cell wall deformation. Three ionogenic groups: amine, galacturonic acid, and phenolic are reportedly involved in cation binding in cell walls (Meychik et al. 2006). In the present study, the significantly increased E_0 values in the sensitive cultivars (Table 3-2) may represent one of the factors that inhibited the root growth. In contrast, the unaffected E_0 of the tolerant cultivars suggested that this parameter may not be a limiting factor for root growth in these studied cultivars under saline conditions. In addition, these results suggest that the mechanical properties of the root cell wall may be related to the cultivar differences in root growth under salinity stress. The turgor pressure of cells, i.e. the driving force for cell elongating, decreases under salinity stress (Rygol and Zimmermann 1990; Ogawa and Yamauchi 2006); therefore, the significance of cell wall elasticity on cell elongation becomes very pronounced under saline conditions.

Hattori et al. (2003) reported an increase in both the extensibility of the root cell wall and root growth in sorghum in response to Si application under drought conditions. Fan et al. (2006) reported that maize root cell wall extensibility and root growth were both inhibited by water deficit stress. Ma et al. (2004) reported that Al directly reduced cell wall extensibility in wheat roots. The decrease in Al-binding with the cell wall has been shown to improve the elastic and

viscous extensibility in tea roots (Safari et al. 2018). Collectively, these previous findings and the findings of the present study indicate that the maintenance of the root cell wall extensibility is important for root growth under abiotic stress conditions. Our findings revealed that the E_0 was only about 1/10 of that of E_{1-3} and almost no significant differences were detected among E_{1-3} across treatments and cultivars (Table 3-2). These results confirmed that the elastic extension of plant roots is mostly determined by E_0 when the Kelvin-Voigt-Burgers model is applied (Tanimoto et al. 2000).

3.5.3 Cell wall extension and viscosity in response to salinity

Different elasticity traits of the cultivars resulted in different elastic and total extension distance in this study (Fig. 3-2A). After accounting for the changes in root thickness (which increased under salinity, Table 3-1), the extension distances of the tolerant cultivars under the saline condition were almost consistent with those under the control conditions (Fig. 3-2B). These findings suggested that the wall elastic property of the tolerant cultivars favoured cell elongation under the saline condition. The elastic extension accounted for approximately one-half to two-thirds of the total extension (Fig. 3-2), thus indicating that the elasticity of the cell wall mostly contributes to the cell extension in wheat. On the other hand, plastic extension accounted for half or less than half of the total extension (Fig. 3-2B) and η_0, which represents the viscosity and determines the plastic extension, was not affected by the salinity in all cultivars (Table 3-2, Fig. 3-2B). Therefore, the elastic properties of the root cell wall are more prominent for root elongation than the plastic properties in wheat.

The viscosity coefficients (η_0, η_1, η_2, η_3,) were in the order of 10^{10}, 10^9, and 10^8 (Table 3-2), and were slightly higher than those reported for sorghum roots (10^{10}, 10^8, 10^8; Hattori et al. 2003), similar to those reported for pea roots (Tanimoto et al. 2000), and much higher than those reported for *Arabidopsis* stems (10^4, 10^5, 10^6; Shigeyama et al. 2016). Previous studies showed that these three cell wall parameters change significantly in response to pH changes

(Tanimoto et al. 2000), Si application (Hattori et al. 2003; Głazowska et al. 2018), Al application (Ma et al. 2004; Safari et al. 2018), and the loss of function of wall glycoside hydrolases (Shigeyama et al. 2016). However, in the present study, the NaCl treatment had no effect on the viscosity (plastic property) of the root cell wall in all cultivars (Table 3-2), except for a slightly high value in Xinchun-31. Tanimoto et al. (2000) suggested that the decrease in viscosity is related to expansin and the removal of other proteins and calcium ions from the cell wall. Hattori et al. (2003) suggested that Si-hemicellulose and Si-pectin conjugates were responsible for the observed changes in root viscosity. Ma et al. (2004) suggested that interference in the binding of new wall materials with old materials increased the viscosity and decreased plastic extension. Shigeyama et al. (2016) reported that the accumulation of free xyloglucan oligosaccharides and the reduced molecular size of xyloglucan in hemicellulose can decrease the viscosity parameters. However, in the present study, the presence of Na^+ did not affect the viscosity coefficient (Table 3-2) and plastic extension in all cultivars (Fig. 3-2B). Since this property and other related plastic extension parameters (e.g. irreversible extension) are also important factors that affect cell elongation, further investigations are needed to clarify how wheat plants maintain this wall property under saline conditions.

3.5.4 Correlations of extension parameters and compositions

The comparable total cell wall amounts under the saline and non-saline conditions in all cultivars showed a stable allocation of carbon assimilation in the wheat cultivars, despite the growing environment (Fig. 3-3). The general decrease in the pectin and increase in the cellulose contents indicated a spatial-temporal change in cells under saline conditions.

To our knowledge, this is the first study to assess the numeric correlations among viscoelastic parameters and cell wall compositions. The positive high correlations among the three kinds of extensions (total, elastic, and plastic) indicated that cell elongation involves both elastic and plastic elongation (Tanimoto et al. 2000; Boudaoud 2010, Table 3-3). The highly

negative correlations between E_0 and the three extensions demonstrated that the elastic property of the cell wall is of great importance for cell elongation and that E_0 may be related to cell wall loosening. The negative correlations between E_0 and the relative pectin and hemicellulose I contents demonstrated the great contribution of these two compositions to cell elastic extension. Although the linkage of pectin-cellulose (Wang et al. 2015) and pectin-xylan (Tan et al. 2013) were reported, the load-bearing points are suggested to be hemicellulose II-cellulose conjunctions (Cosgrove 2018). Therefore, higher amounts of pectin and hemicellulose I would be expected to benefit cell elongation.

Table 3-3 Correlation analysis of the extension, viscoelastic parameters, and composition of the root cell wall and root growth in wheat

	Total extension	Elastic extension	Plastic extension	E_0	η_0	Pectin	Hemi-cellulose 1	Hemi-cellulose 2	Cellulose	Relative growth
Total extension	1									
Elastic extension	0.961**	1								
Plastic extension	0.898**	0.746*	1							
E_0	-0.848*	-0.802*	-0.824*	1						
η_0	-0.190	0.005	-0.603	0.842**	1					
Pectin	0.848**	0.848**	0.716*	-0.710*	-0.368	1				
Hemicellulose I	-0.496	0.413	0.586	-0.775*	-0.830*	0.167	1			
Hemicellulose II	0.182	0.251	0.033	0.060	0.046	0.351	-0.471	1		
Cellulose	-0.860*	-0.849*	-0.757*	0.830*	0.686	-0.832*	-0.431	-0.493	1	
Relative growth	-0.941**	-0.956**	-0.774*	0.424	-0.036	-0.866**	0.149	-0.398	0.592	1

Significant correlations are indicated by asterisks (*$P < 0.05$, **$P < 0.01$)
E_0 and η_0 indicate the elastic and viscous properties of the root cell wall, respectively.

The negative correlation between the relative pectin and cellulose contents and the opposite correlations of these two compositions with the extension parameters (E_0, total, plastic, and elastic extension, Table 3-3) indicate that the deposition of cellulose to the growing cell wall restricts the elongation of the cell while higher amounts of pectin improve cell elongation. This notion is consistent with the report by An et al. (2014b), who showed that an increase in the pectin content induced by Ca application enhanced root growth in soybean. Contrasting effects of pectin and cellulose on cell wall extension have been reported for white sprunce (Renault and Zwiazek 1997). With the exception of the mechanical properties, the functions of pectin in Na-binding, water retention (Jung et al. 2019), and pH adjustment (Cosgrove 2018) may all comprehensively benefit plant cell growth under saline conditions.

A high positive correlation between E_0 and η_0 reveals interactions between the elastic and viscosity properties of the cell wall. Previous reports, although not statistically supported, also showed a positive correlation between these two parameters (Hattori et al. 2003; Ma et al. 2004). In the present study, the final root growth under the saline condition was determined to be positively correlated with the total, elastic, and plastic extensions, as well as the relative pectin content (Table 3-3). These results revealed the significance of the root cell wall properties and the special role that pectin plays in root growth under salinity stress. The present study did not show any correlation between hemicellulose II and any other parameters, possibly because the amount of hemicellulose II was too low to produce the statistical results (Table 3-3). Nevertheless, the decreased hemicellulose II content in the tolerant cultivars implied a decrease in the load-bearing points in the cell wall, which may enhance the wall extensibility.

Based on the growth processes and dynamics of the cell wall (Cosgrove 2018), our results implied that the loosening of root cell wall under saline conditions (with reduced turgor pressure) was largely depressed in the sensitive cultivars but maintained to some extent in the tolerant cultivars. This wall loossenning corresponded to the elastic extension. When the root cell wall loosens, new wall materials fill in the space or bind to the old wall. These materials improve the viscosity and their levels correspond with the plastic nature, i.e. the final elongation, of the root region. Present study revealed the regulation role of cell wall in root growth. Cultivar difference in salt tolerance may be related with the property of root cell wall. Further studies on the changes, constitutions, and functions of the chemical compositions with regards to the cell wall extension in various crops are needed to fully understand the role of cell walls in root growth under abiotic stresses.

Chapter 4. Specific expression of expansins in response to apoplastic pH under salinity stress

4.1 Abstract

Plant salt tolerance is associated with a high rate of root growth. Although root growth is governed by cell wall and apoplastic pH, the relationship between these factors in the root elongation zone under salinity stress remains unclear. Herein, apoplastic pH, pH- and expansin-dependent cell wall extensibility, and expansin expression in the root elongation zone of salt-sensitive (Yongliang-15) and -tolerant (JS-7) cultivars under salinity stress were assessed. A six-day 80 mM NaCl treatment significantly reduced apical-root apoplastic pH in both cultivars. Under 0 mM NaCl treatment, the optimal pH for cell wall loosening was 6.0 in the salinity-tolerant cultivar and 4.6 in the salinity-sensitive cultivar. Under 80 mM treatment, a pH of 5.0 mitigated the cell wall stiffness caused by salinity stress in the salinity-tolerant cultivar but promoted cell wall stiffening in the salinity-sensitive cultivar. Salinity stress altered expansin expression and differentially affecting cell wall extensibility under pH 5.0 and 6.0. *TaEXPA8* might be relative to cell wall loosening at pH 5.0, whereas *TaEXPA5* relative to cell wall loosening at pH 6.0. These results elucidate the relationship between expansin and cell wall extensibility in the root elongation zone, with important implications for enhancing plant growth under salinity stress.

4.2 Introduction

Under abiotic stress, the plants primary cell wall protects cell integrity and regulates cell expansion and division (Cosgrove 2018). When plants are exposed to salinity stress, the cell wall, as the outmost layer of cells, is the first line of defense. Salinity stress alters cell wall structure (Koyro 1997) and composition (Byrt et al. 2018), factors closely associated with cell wall extensibility. Salinity stress causes changes in ion homeostasis and transportation across the cell wall. The roots can extrude sodium ions (Na^+) out of cytosol (Munns et al. 2020). Na^+ extrusion alters the root apoplastic microenvironment, which shifts the apoplastic pH away from the range that favours cell wall loosening (Byrt et al. 2018).

Studies on changes in apoplastic pH under salinity stress have focused mainly on the leaves. Transient leaf-apoplast alkalization under salinity stress has been reported in the field bean (Felle and Hanstein 2002), barley (Felle et al. 2005), and maize (Geilfus et al., 2017). Further, an eight-day NaCl treatment induced apoplastic acidification in maize leaves (Zörb et al. 2015). However, there is limited information regarding the long-term response of root apoplastic pH to salinity stress. Short-term apoplastic alkalization in the root under salinity stress has been reported in *Arabidopsis* (Gao et al. 2004), although the authors did not clarify whether the apoplastic alkalization occurred in the root elongation zone or in the mature zone.

Apoplastic pH regulates both cell wall composition and extensibility. pH-dependent cell wall extensibility is explained according to the "acid growth theory" (Rayle and Cleland 1992): Apoplastic acidification triggers cell wall loosening, resulting in cell elongation and expansion. In *Arabidopsis*, apoplastic pH steers root growth (Barbez et al. 2017), promotes cell differentiation (Pacifici et al. 2018), and regulates cell shape (Dang et al. 2020). The optimal pH for cell wall loosening differs between shoots and roots. In pea (*Pisum sativum*) grown under hydroponic conditions, pH 3.0 buffer induced the maximum apical-root extensibility, compared with pH values of 4.0–8.0 (Tanimoto et al. 2000). In wheat coleoptiles, the optimum

pH for cell wall loosening is 4.0–4.5 (Gao et al. 2008). In maize, root cell wall extensibility decreased under low water potential (Wu et al. 1996). Low apoplastic pH (pH 4.5) increased cell wall extensibility in the apical 5 mm root but decreased in the adjacent 5 mm (Wu and Cosgrove 2000). Apoplastic acidification in the root elongation zone does not increase maize growth under salinity stress (Zidan et al. 1990). These results indicate the low apoplastic pH may not induce high cell wall creep under abiotic stress.

Apoplastic pH-dependent cell wall loosening is facilitated by expansins (Cosgrove 2005). Cellulose-xyloglucan-cellulose conjunctions form the main load-bearing structures in the cell wall (Park and Cosgrove 2012); expansin can bind to hydrophilic regions on these conjunctions, unzipping the covalent bonds and loosening the cell walls (Cosgrove 2018). Expansin genes are abundant in plants. In the wheat genome, the expansin gene superfamily contains over two hundred expansin genes, more than in rice, *Arabidopsis*, and tomato (Han et al. 2019). Many literatures have shown that expansin plays important roles under abiotic stress. In wheat. Drought stress increases cell walls susceptibility to exogenous expansin treatment (Zhao et al. 2011). Under low-temperature stress, the expansin gene *TaEXPA8* is highly expressed in a cold-tolerant wheat cultivar (Zhang et al. 2018); in *Arabidopsis*, its overexpression improves cold tolerance (Peng et al. 2019). In wheat under salinity- and PEG-induced stress, expansin gene expression differs between the leaves and roots (Han et al. 2019), and in transgenic tobacco, overexpression of wheat coleoptile expansin genes, such as *TaEXPA2* (Chen et al. 2017) and *TaEXPB23* (Han et al. 2012) enhances root growth and salt tolerance. These studies believe that, under various types of abiotic stress, expansin closely related to phytohormone and ROS. In contrast, relationships between expansin expression and pH-dependent cell wall extensibility have not been widely studied.

Therefore, herein, the aim of this study was to clarify how the relationship between apoplastic pH and expansin affects cell wall extensibility under salinity stress. Using salinity-

sensitive and salinity-tolerant wheat cultivars, changes in apical-root apoplastic pH and cell wall extensibility, using buffers with different pH values and exogenous expansin treatments, and expansin gene expression were examined. Understanding the relationship between apoplastic pH and pH-dependent cell wall extensibility under salt stress is of great importance, not only to elucidate the mechanism underlaying plant growth regulation under salinity stress, but also to improve the screening or breeding of stress-adapted crop plants.

4.3 Materials and Methods

4.3.1 Cultivation of wheat seedlings

Two spring wheat cultivars, Yongliang-15 (YL-15) and JS-7, that differ in salinity tolerance were used. Seeds of the two cultivars were surface sterilized in 70% ethanol for 5 min, then soaked in distilled water overnight. Twenty seeds were placed in a line on a sheet of filter paper, which was then placed in a 24 × 34 cm Ziploc bag and wetted with 50 ml distilled water. The seeds sprouted in growth chambers (MLR-350 HT, SANYO, Moriguchi, Japan) at 28 °C (24 h dark). Starting two days later, when the roots reached ca. 1.5 cm, 1/12 strength Hoagland solution, containing 0 or 80 NaCl treatments, was applied to the roots every 2 d. Light was provided at 2,000 lx (16 h light / 8 h dark), and the chamber temperature was constant at 25 °C.

4.3.2 Apoplastic pH

The HPTS (8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt hydrate) were used to investigate apoplastic pH in the elongation and mature zones at a cellular resolution. HPTS, an extracellular pH indicator, has low toxicity because it does not penetrate the cell membrane (Han and Burgess 2010); it has been used to assess cell wall apoplastic pH in *Arabidopsis* roots and petals (Barbez et al. 2017; Dang et al. 2020).

For imaging analysis of apoplastic pH in the root elongation zone, 50 ml of 0 mM or 80 mM NaCl solution (1/12 Hoagland solution, pH 6.5), supplemented with 1 mM HPTS, were applied to the two-day-old seedlings. After irrigating the plants with the salt treatments containing HPTS, six-days-old root of plants were sandwiched between a cover glass and a 35-mm petri dish (with a 20 mm micro-well; Matsunami Glass, Osaka, Japan). Root imaging was performed using a confocal laser scan microscope (FV10-ASW; Olympus, Tokyo, Japan). Fluorescence signals for the protonated HPTS (excitation at 405 nm) and deprotonated HPTS (excitation at 473 nm) were detected using a 60× oil-immersion objective lens. The ratiometric image was obtained by dividing the signal intensity of the 473-nm channel by that of the 405-nm channel, for each pixel. For calibrating the HPTS dye, the wheat roots were stained in medium of a given pH between 4.0 and 7.0, supplemented with 1 mM HPTS, for 30 min. A best-fitting regression method was used to plot the calibration curve. Image analysis was performed using Fiji software (https://fiji.sc/) and a customized macro script (Barbez et al. 2017), with a slight modification. The experiments were performed using at least six biological replicates.

4.3.3 Expansin extraction

The whole protein from the cell were used because the previous study showed the protein extracted from the cytosol did not change the cell wall extensibility (McQueen-Mason et al. 1992). Attempts to extract expansin from cell wall preparations of wheat root tips by extracting with a high salt buffer were unsuccessful. Therefore, the expansin extraction was performed following Harrison et al. (2001), with a slight modification. Roots (20 g) were homogenized in a blender with liquid nitrogen and macerated further with 100 ml of extraction buffer (comprising 10 mM 3-[N-morpholino] propanesulphonic acid (MOPS)–NaOH buffer at pH 7.0, 0.5% (w/v) cetyltrimethylammonium bromide, and 30% (w/v) glycerol), until the mixture reached ambient temperature (24 °C). The extraction buffer was then collected and filtered

through Miracloth (Merck Millipore, MA, US). Three volumes of precooled acetone were added to the extract, which was then incubated at −20 °C overnight. The mixture was centrifuged at 5 000 ×*g* for 10 min at 4 °C, the supernatant was discarded, and the protein pellet washed once with three volumes of acetone (−20 °C) before freeze-drying. The dried pellets were stored at −20 °C. Protein was assayed using the Bradford method, using a commercial kit (TaKaRa, Shiga, Japan). Before applying to the root segments, each expansin pellet was dissolved in citrate-phosphate buffer. After being centrifuged at 5 000 ×*g* for 10 min at 4 °C, the supernatant solution was diluted to diluted to 100 μg/ml.

4.3.4 Root extensibility

As the root extensibility experiments and expansin extraction required many roots (more than 1,000), the experimental period was extended to collect enough samples. Roots tips of ten-day-old seedlings grown in 0 mM or 80 mM NaCl solutions (1/12 Hoagland solution, pH 6.5) were used to measure changes in cell wall extensibility, at various buffer-pH values and expansin concentrations. Root-tip extensibility was determined following a method developed by Tanimoto et al. (2000). The root extensibility experiments had two parts: Experiment I assessed changes in the cell wall extensibility of root segments at pH ranging from 3.0 to 6.0; experiment II assessed the effects of exogenous expansin on cell wall extensibility using buffer at pH 5.0 or pH 6.0.

In experiment I, root segments of 10 mm from the root tip were prepared by placing them in boiling methanol (80 °C) for 3 min, according to McQueen-Mason et al. (1992). Methanol-boiling treatment denatures the cells but partly preserves expansin activity, whereas water-boiling denatures all expansins (McQueen-Mason et al. 1992). Before the cell wall extensibility measurement, the methanol-boiled root segments were hydrated with distilled water at ambient temperature (24 °C) for 15 min, then incubated in citrate-phosphate buffer, with pH ranging

from 3.0 to 6.0, for more than 30 min under ambient temperature (24 °C). In experiment II, the root tips were boiled in water, to entirely inactive the expansin. To obtain the exogenous expansin for the later experiments, four sets of expansin samples were extracted, one from each cultivar grown under each salinity treatment. After being hydrated with distilled water, the water-boiled root segments were incubated with exogenous expansin at pH 5.0 or 6.0 (citrate-phosphate buffer) for more than 30 min.

After being treated with various pH buffers or exogenous expansin concentrations, the extensibility parameters—the elasticity modulus (E_0) and viscosity coefficient (η_N)—of the root specimens under a constant tensile force were measured. Before the root specimen was mounted between clips, the diameter of the root at ca. 5 mm from root tip was measured using a microscope (Vitiny UM12, Taiwan, China). The extensibility of the root region between 3–8 mm from the root tip measured using a creep meter (Yamaden RE2-33005C, Tokyo, Japan). A tensile force of 0.05 N was found to be optimal for obtaining the typical extensibility curve, based on a previous study (Tanimoto et al. 2000) and our preliminary tests. Roots were stretched for 5 min and then released for 5 min. The experiments were performed using at least 19 biological replicates. Details regarding the root extensibility measurements are reported by Tanimoto et al. (2000).

4.3.5 Expansin expression and Co-expression analysis

To evaluate changes in expansin expression in response to salinity stress, the total mRNA from the root tips of YL-15 and JS-7 plants treated with 0 mM or 80 mM NaCl were extracted. The transcripts of selected expansin genes that are highly expressed in wheat root tips were then analysed, based on a previous study (Lin et al. 2005).

The total mRNA from 60–80 mg of wheat root tips that had been subjected to 0 mM or 80 mM NaCl treatment was extracted using a NucleoSpin® RNA kit with rDNase (TaKaRa, Shiga,

Japan). cDNA was synthesized using a PrimeScript® RT Reagent Kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. Specific primer sequences for the expansin genes were designed using the Triticeae Multi-omics Center primer server (http://202.194.139.32/PrimerServer), and specificity was checked by blasting the sequences against IWGSC RefSeq annotation v1.1 (Appels et al. 2018). Actin was used as the reference gene, and actin primers were from a previous study (Zhu et al. 2016). The qRT-PCR reaction mixture included TB Green® Premix Ex Taq™ II (Takara, Shiga, Japan). The qRT-PCR conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 15 s. Three biological replicates were used for each sample. The sequence data were analysed using the $2^{-\Delta\Delta CT}$ method, according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al. 2009).

TaEXPA5-A, -B, -D (gene ID: TraesCS3A02G165900, TraesCS3B02G199900, TraesCS3D02G175800) and *TaEXPA8-A, -B, -D* (gene ID: TraesCS3A02G187600, TraesCS3B02G217000, TraesCS3D02G191300) were examined using the knowledge network generated by KnetMiner (http://knetminer.rothamsted.ac.uk; Hassani-Pak et al. 2020). The expansin network includes both wheat-specific information sources and cross-species information, from *Arabidopsis*.

4.3.6 RNA-seq expression analysis

The publicly available RNA-seq data generated from the bread-wheat cultivars Chinese Spring and Qing Mai 6 were used to study the wheat expansin gene expression (GenBank accession SRP062745; Zhang et al. 2016). The expansin expression data were obtained from the Triticeae Multi-omics Center (http://202.194.139.32/expression/index.html). Euclidean-distance cluster analysis of the RNA-seq data was conducted using TBtools (Chen et al. 2020).

4.3.7 Statistical analysis

Statistical tests were performed using IBM Statistics 21 (SPSS Inc., Chicago, IL, USA). Data-distribution normality was analysed using the Shapiro-Wilk test. E_0 and η_N were normalized via log-transformation. For pairwise comparisons, statistical differences were detected using a Student's *t*-test. For comparing apoplastic pH among the zones, cultivars, and treatments, the fluorescence intensity ratio (the intensity at 473 nm divided by the intensity at 405 nm) data were analysed using a one-way ANOVA and Duncan's new multiple range test. For correlation analysis, Pearson correlation coefficient between the traits were calculated using R package, PerformanceAnalytics and fitted with linear regression. To detect statistical differences between the groups, in terms of root segments, expansin expression, and pH, a three-way ANOVA with Bonferroni post-hoc tests were performed.

4.4 Results

4.4.1 Root growth

Two wheat cultivars, Yongliang-15 (YL-15; salinity-sensitive) and JS-7 (salinity-tolerant), were treated with NaCl at 0 mM and 100 mM. After 30 d of salinity stress, YL-15 showed worse chlorosis than JS-7. In the cultivation experiment using filter-paper, JS-7 showed significantly faster root growth than YL-15, under salinity stress (Fig. 4-1A, B, C). After 4 d of 80 mM NaCl treatment, JS-7's root growth recovered and remained relatively high, whereas the root growth rate of YL-15 was low (Fig. 4-1A). Because the root-growth rates of the two cultivars differed significantly at day 6 of the salinity treatment (Fig. 4-1C), the apoplastic pH in the roots after 6 d of 80 mM NaCl treatment was analysed to investigate the effects of Na^+ ions on apoplastic pH.

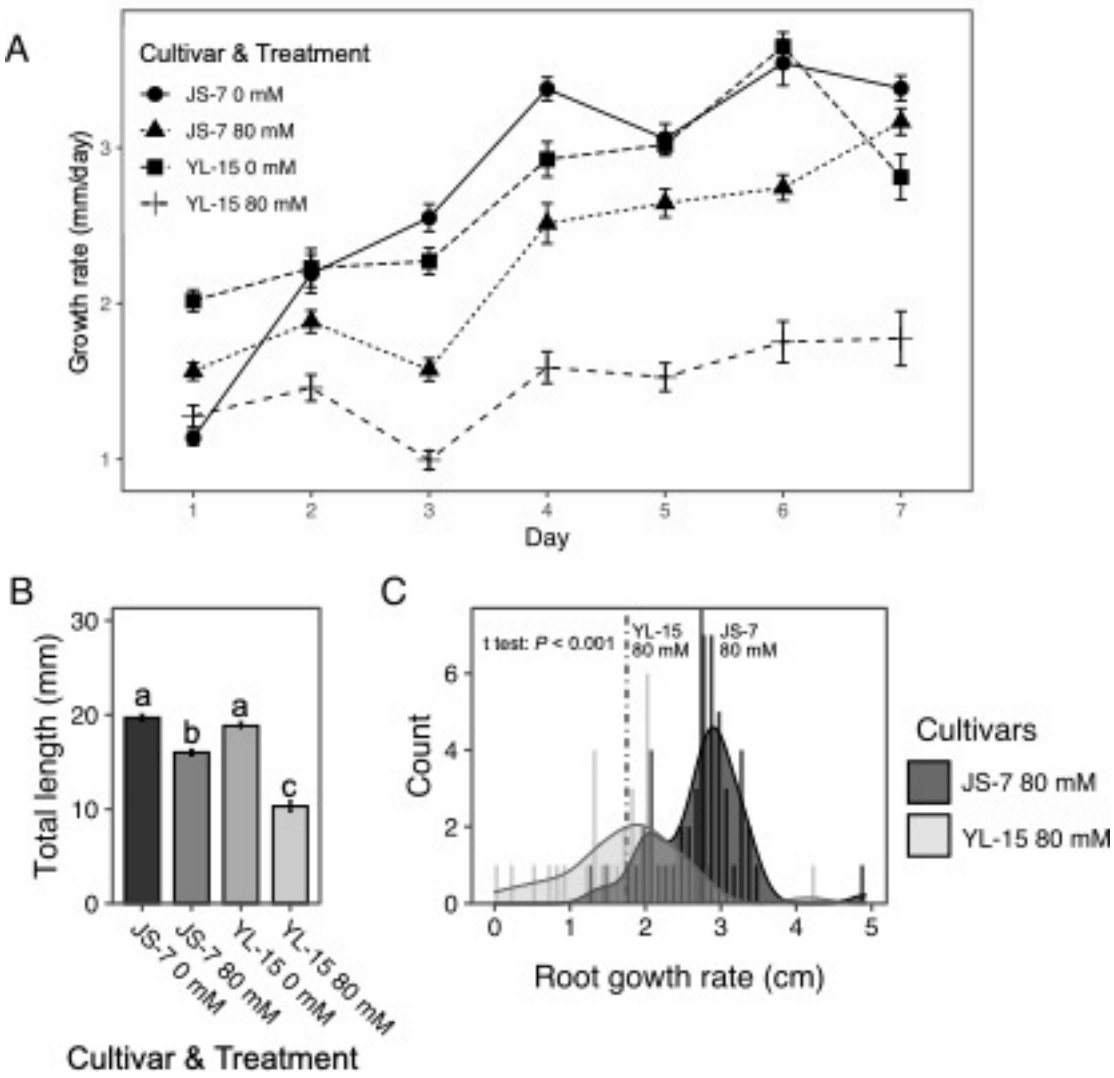

Figure 4-1. Root-growth rate and total length of the Yongliang-15 (YL-15) and JS-7 wheat cultivars, under the control and salinity-stress treatments.

Root-growth rate of YL-15 and JS-7 under seven-day 0 mM and 80 mM NaCl treatments. Growth rate was recorded daily. (B) Total root length of YL-15 and JS-7 under seven-day 0 mM and 80 mM treatments. The data are shown as the mean ± SE (*n*: 37–51). (C) Histogram distribution of root-growth rate of two cultivars after 6-d 80 mM salt treatment.

4.4.2 Apoplastic pH

The 473/405 nm intensity ratio increased with the pH value of the medium (Fig. 4-2A, B). The ratio values were used to plot a calibration curve over the range of physiological pH of the root cell wall (pH 4.0 to pH 7.0; Fig. 4-2C). The absorption spectra of HPTS at pH-values from 4.09 to 7.9 with λex 405 nm and 473 nm was analysed. The results showed the fluorescent intensity of HPTS at 473 and 405 nm can present the changes in pH from 4.2 to 7.0.

Moving away from the root tips, the apoplastic pH decreased, and the region 1 mm from the root tips showed the lowest apoplastic pH, in both cultivars and under both NaCl treatments (Fig. 4-2D). Therefore, in this study, pH at 1 mm from the root tip was measured as representative of the elongation zone and pH at 5 mm from root tip was as representative of the mature zone.

After treating wheat roots with 0 mM or 80 mM NaCl supplemented with 1/12 strength Hoagland solution for 6 d, apoplastic pH in the root elongation and mature zones was measured. 473/405 nm intensity ratio was lower in the root elongation zone than in the mature zone in both cultivars under both treatments (Fig. 4-2E, F), and this ratio was lower under the 80 mM treatment than under control, in both zones and cultivars (Fig. 4-2A, B). Under the 80 mM NaCl treatment, intensity ratio decreased from 1.53 (equivalent to pH ~6.28) to 0.48 (pH ~5.3) in the elongation zone (Fig. 4-2E), and from 4.23 (pH ~6.9) to 1.72 (pH ~6.36) in the mature zone, in both wheat cultivars (Fig. 4-2E). In summary, salinity stress induced apoplastic acidification in the elongation and mature zones in both cultivars.

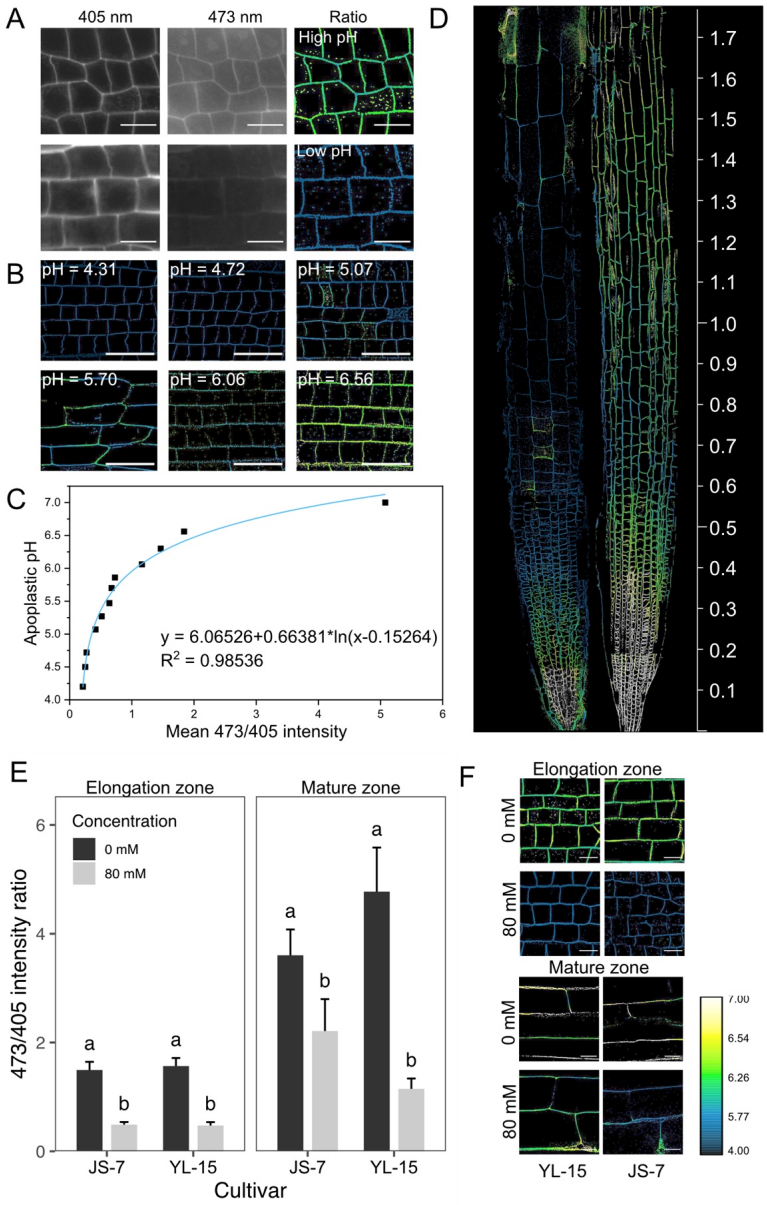

Figure 4-2. HPTS staining of wheat apical roots, HPTS calibration and apoplastic pH in the root elongation and mature zone of Yongliang-15 (YL-15) and JS-7 wheat cultivars, under 0 mM and 80 mM NaCl treatments.

(A) HPTS staining of root cells. (Left) Protonated (acidic) version of HPTS (λ_{ex} 405 nm; λ_{em} 430 nm). (Middle) Deprotonated (basic) version of HPTS (λ_{ex} 473 nm; λ_{em} 520 nm). (Right) Ratiometric image: For each pixel, the 473 intensity is divided by the 405 intensity. (B) HPTS calibration. Apoplastic epidermal root-meristem 473/405 values, of seedlings incubated for 30 min in citrate-phosphate buffer, pH 4.2–7.0. (C) Regression analysis-derived equation enabling calculation of apoplastic pH from the obtained 473/405 values. (D) HPTS-stained root tip of six-day-old seedling under 0 mM and 80 mM NaCl treatments. The color key shows the 475/405 intensity ratio. Scale bars: 20 µm (A) and 50 µm (B). (E) Analysis of apoplastic pH in the elongation (left) and mature (right) zones under 0 (green bars) and 80 mM (orange bars) NaCl treatments. The data are shown as the mean ± SE (*n*: 6–9 roots per data point). The different lowercase letters above the bars identify groups that differ significantly ($P < 0.05$). (F) HPTS staining of root cells in the elongation and mature zones under 0 mM and 80 mM treatments, respectively. The color key indicates pH. Scale bars: 20 µm.

4.4.3 Cell wall extensibility

Byrt et al. (2018) hypothesized that salinity stress alters apoplastic pH, thereby inhibiting cell wall loosening. To analyse differences in cell wall extensibility between the two cultivars,

the effects of salinity stress and pH on the cell wall elasticity modulus (E_0), extensibility coefficient (η_N) and extension (elastic and plastic extension) of the root tips were assessed. In both cultivars, E_0 was significantly higher under the 80 mM NaCl treatment than the control (Fig. 4-3A). When apoplastic pH was from 4.0 to 5.0, the increments in E_0 in YL-15 were greater than those in JS-7, indicating that the root cell walls of YL-15 were stiffer at low apoplastic pH than those of JS-7 under the 80 mM treatment (Fig. 4-3C). The differences in E_0 and η_N obtained by varying the buffer pH indicate that, for the 0 mM NaCl treatment, the optimal pH values for cell wall loosening were 4.6 in the apical roots of YL-15 and 6.0 in segments of JS-7 (Fig. 4-3A, B); for the 80 mM NaCl treatment, a pH of 4.0–5.0 caused cell wall stiffening in the apical roots of YL-15, whereas this pH range loosened the cell walls in JS-7 (Fig. 4-3A, B). Elastic extension and creep of root tips confirmed this result. For the 80 mM NaCl treatment, a pH of 4.0–5.0 caused more cell wall creep in the apical roots JS-7 compared with YL-15 (Fig. 4-3D). Between pH 4.0 and 5.0, both JS-7 and YL-15 root segments showed less elastic extension under the 80 mM treatment than 0 mM treatment (Fig. 4-3D). Increases in E_0 and η_N are associated with reductions in cell wall elastic extension and creep, respectively (Fig. 4-3E). In summary, salinity stress stiffened the cell walls and altered the pH-dependent cell wall extensibility in the apical roots of both cultivars. In contrast, the apoplastic acidification inhibited cell wall loosening in YL-15 but favoured it in JS-7.

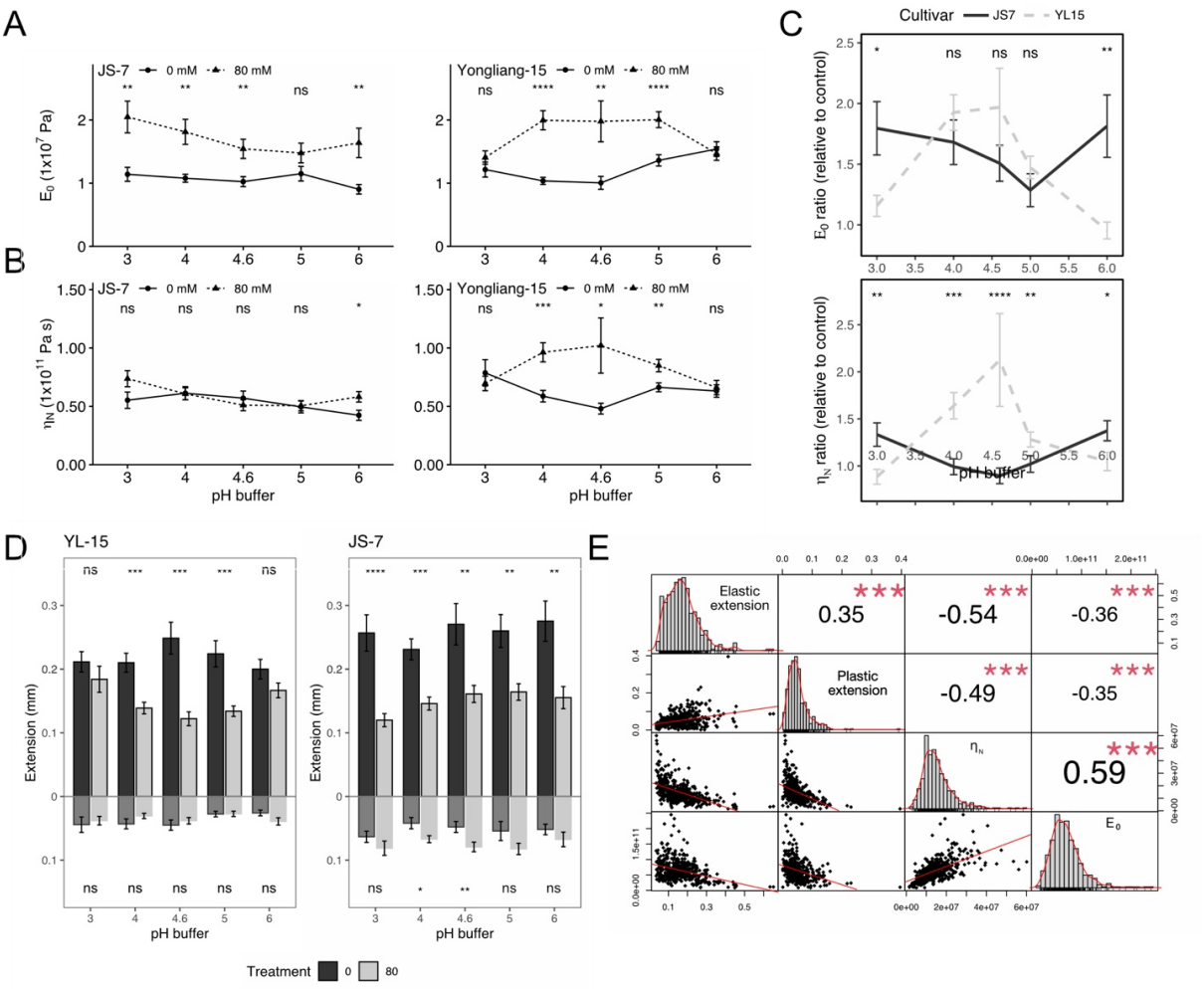

Figure 4-3. Cell wall elasticity parameter (E_0), creep coefficient (η_N), extension, and correlation between the parameters in the apical roots of Yongliang-15 (YL-15) and JS-7 wheat cultivars, under the 0 mM and 80 mM NaCl treatments.

E_0 (A) and η_N (B) of the cell wall in the root tips (3–8 mm from root tip) of the two wheat cultivars, at pH 3.0–6.0. The root tips were collected after 10-day 0 mM (solid line) and 80 (dash line) mM NaCl treatments in JS-7 (left) and YL-15 (right). The data are the mean ± SE (*n*: 19–34). Asterisks indicate a significant difference between the treatments (ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). (C) Relative E_0 (top) and η_N (bottom) value of the cell wall under 80 mM treatment. Asterisks indicate a significant difference between the wheat cultivars (ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). (D) Elastic extension (above zero) and creep (below zero) of the cell wall in the root tips (3–8 mm from root tip) of the two wheat cultivars, at pH 3.0–6.0. Black and grey bars indicate extension of the root cell wall that grown under 0 and 80 mM NaCl treatment, respectively. The data are the mean ± SE (*n*: 19–34). Asterisks indicate a significant difference between the wheat cultivars (ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). (E) Correlation between cell wall parameters (E_0 and η_N) and extension. Upper shows the Pearson coefficient and significance. Lower shows the linear regression between parameters and extension. Middle shows the histogram distribution of parameters and extension, respectively. Asterisks indicate a significant correlation between parameters.

4.4.4 Cell wall extensibility and expansin expression

The expansin-denatured root segments and extracted four sets of protein samples containing expansins (from the two cultivars and two salinity treatments, respectively) were collected. The loosening effects of exogenous expansins on cell walls were assessed under pH 5.0 and 6.0.

The apical-root cell walls of two cultivars showed different susceptibilities to the four sets of expansin samples (Fig. 4-4A, B). To assess the pH-dependent effects of four sets of expansins, all apical roots were combined and the cell wall extensibilities under pH 5.0 and 6.0 were analysed. In general, the expansin extracted from the root of JS-7 grown under the 80 mM NaCl treatment (JS-7 80 mM) induced the lowest E_0 and η_N at pH 5.0, whereas that extracted from YL-15 80 mM induced the highest E_0 and η_N at the same pH (Fig. 4-4C). Between pH 5.0 and 6.0, no obvious changes were found in root segments grown under 80 mM treatment (Fig. 4-4D). Three-way ANOVA revealed significant effects on cell wall extensibility of expansin, root segment, and the interaction between expansin and buffer pH, but not of the interaction between root segments and buffer pH (Table 4-1). The differences in cell wall extensibility in response to exogenous expansin treatment reveal that expansin determined pH-dependent cell wall extensibility. When apoplastic pH was at 5.0, expansin from YL-15 80 mM induced cell wall stiffness, whereas expansin from JS-7 80 mM induced cell wall loosening.

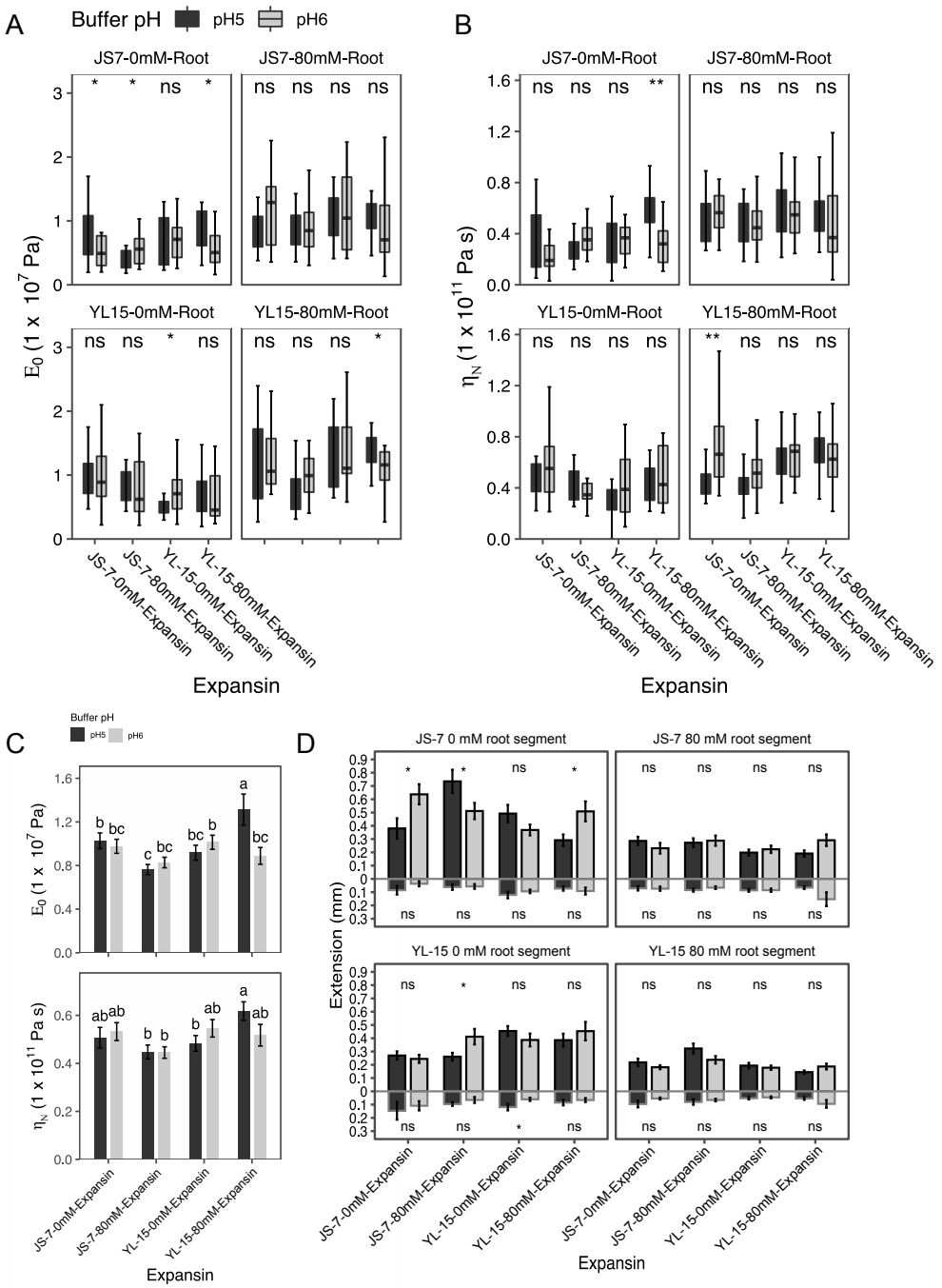

Figure 4-4. Effects of exogenous expansins on cell wall elasticity (E_0) and creep (η_N) in the root tips of two wheat cultivars, Yongliang-15 (YL-15) and JS-7.

E_0 modulus (A) and η_N coefficient (B) of four sets of root cell walls treated with the four sets of expansin samples, at pH 5.0 (black box) and pH 6.0 (grey box) buffer. The root segments and expansin samples were separated to two cultivars and two treatment, respectively (*n*: 15–30). (C) E_0 and η_N of all apical roots treated with four sets of expansin samples in pH 5.0 or pH 6.0 buffer. The data are the mean ± SE (*n*: 69–79). The different lowercase letters above the bars identify groups that differ significantly ($P < 0.05$). (D) Elastic extension (above zero) and creep (below zero) of root cell walls treated with the four sets of expansin samples, at pH 5.0 and pH 6.0. Expansins were extracted from the Yongliang-15 (YL-15) and JS-7 cultivars under the 0 mM and 80 mM NaCl treatments. Asterisks indicate a significant difference between the wheat cultivars (ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$).

The expansin expression in the two cultivars under salinity stress was measured. Compared with expansin expression in the control, expression was inhibited in the YL-15 80 mM root tips (except for *TaEXPA5* and *TaEXPA9* expression); in contrast, in JS-7 80 mM, *TaEXP5* and *TaEXP8* showed elevated expression and *TaEXPA7* and *TaEXPA9* maintained the same expression level. Under the 80 mM NaCl treatment, the cultivars both showed reduced expression of *TaEXPA3*, *TaEXPA6*, *TaEXPB1*, *TaEXPB7*, and *TaEXPB10*; the reductions did not differ significantly different between the cultivars (Fig. 4-5). Under salinity stress, elevated *TaEXPA5* and *TaEXPA8* expression may mitigate cell wall stiffness and enhance root growth in JS-7, the salinity-tolerant cultivar, via apoplastic acidification (Fig. 4-5). In summary, the roots of JS-7 and YL-15 differentially expressed the expansin genes under salinity stress, which altered the optimal pH for cell wall loosening.

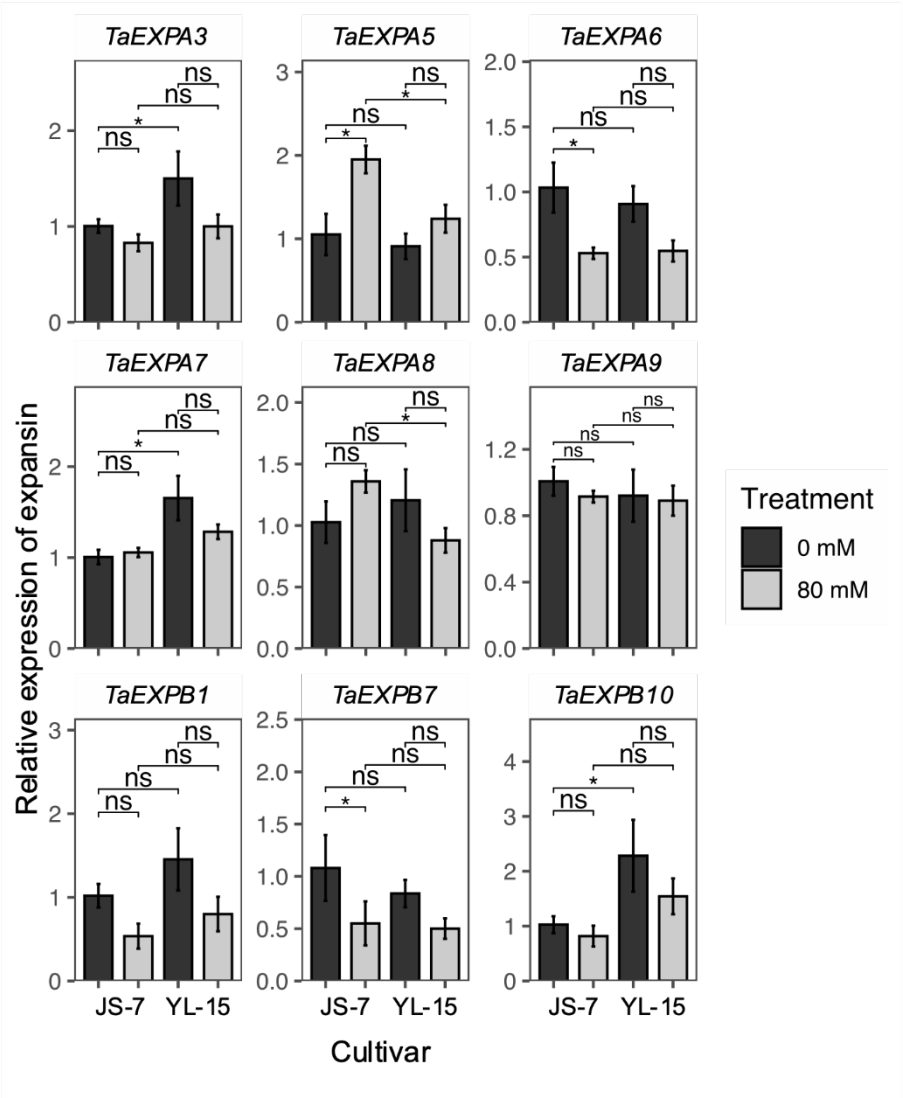

Figure 4-5. Expression profiling of expansins in wheat roots under 80 mM NaCl stress.

The relative expression levels reflect expansin expression under the 80 mM treatment relative to that under 0 mM treatment, in the Yongliang-15 (YL-15) and JS-7 cultivars. Error bars: SEs of three biological replicates. Statistically significant differences between YL-15 and JS-7 were calculated using Student's *t*-tests: ** $P < 0.01$.

Table 4-1. Three-way ANOVA of the effects of root segment, expansin, and buffer pH on the cell wall elasticity parameter (E_0) and extensibility coefficient (η_N).

Dependent Variable: E_0

Source	df	F-value	Probability
Root segment	3	33.500	0.000
Expansin	3	7.083	0.000
Buffer pH	1	1.772	0.184
Root segment * Expansin	9	3.041	0.001
Root segment * Buffer pH	3	0.277	0.842
Expansin * Buffer pH	3	5.778	0.001
Root segment * Expansin * Buffer pH	9	1.253	0.260

Dependent Variable: η_N

Source	df	F-value	Probability
Root segment	3	25.897	0.000
Expansin	3	4.951	0.002
Buffer pH	1	0.003	0.953
Root segment * Expansin	9	2.089	0.029
Root segment * Buffer pH	3	0.976	0.404
Expansin * Buffer pH	3	2.196	0.088
Root segment * Expansin * Buffer pH	9	1.089	0.369

After immersion in exogenous expansin at pH 5.0 or 6.0 for 30 min, the root segments (3–8 mm from the root tip), of plants that had been subjected to 0 mM and 80 mM NaCl treatments, were stretched under 0.05 N tensile force. The exogenous expansins were extracted from the JS-7 and YL-15 wheat cultivars under 0 mM and 80 mM NaCl treatments.

4.5 Discussion

4.5.1 Root apoplastic pH under salinity stress

Apoplastic pH steers cell elongation (Barbez et al. 2017), drives cell differentiation (Pacifici et al. 2018), and regulates cell shape (Dang et al. 2020). For both cultivars, the apoplastic pH was ~5.3 under 80 mM NaCl condition, whereas the pH was at ~6.3 under 0 mM treatment, indicating that long-term salinity stress significantly acidified the apical-root apoplast. However, previous studies have found that the salinity stress induced transient alkalization in leaf apoplasts—from pH 4.2 to 4.5 in faba bean (*Vicia faba*; Geilfus and Mühling 2012) and from pH 4.7 to 5.1 in maize (Geilfus et al. 2015)—and in root apoplasts, from pH 6.4 to 6.8, in *Arabidopsis* (Gao et al. 2004). Leaf apoplastic alkalization plays an important role in stomatal movement (Geilfus et al. 2017). In *Arabidopsis* roots, apoplastic alkalization may be associated with early growth arrest in response to the salinity stress (van Zelm et al. 2020).

In wheat root, the extent of apoplastic acidification caused by long-term salinity stress may be affected by ion-channel functioning and interactions between Na^+ ions and the cell walls. Ion-channel proteins, such as SOS1 and PM-H^+-ATPase, together pump more than 95% of Na^+ ions back into the rhizosphere (Munns et al. 2020). In this Na^+-extrusion process, PM-H^+-ATPase is activated to polarize the cell membrane. SOS1 is then activated to pump Na^+ ions out of cytosol. Further, under salinity stress, Na^+ ions interact with the polyglucuronic acid (PGA) in cell walls and release the H^+ from the PGA carboxyl groups (Feng et al. 2018). These findings imply that salinity stress causes apoplastic acidification in roots, especially in root tips, where the pH determines the growth rate of roots.

4.5.2 Apoplastic pH in relation with cell wall extensibility

The extensibility parameters, E_0 and η_N, were higher in both cultivars under the 80 mM treatment, indicating the salinity stress stiffened the cell walls in both cultivars (Fig. 4-3A, B).

Thus, the root-growth rates and final lengths of the two cultivars were lower under salinity stress than under the control (Fig. 4-1A, B). Under the 80 mM treatment, JS-7 had faster root growth than YL-15, suggesting that JS-7 has superior cell-elongation ability under salinity stress. Interestingly, salinity stress reduced apoplastic pH in the apical roots of both cultivars. However, apoplastic acidification under salinity stress favoured cell wall loosening in JS-7 but had the opposite effect in YL-15. The root growth of YL-15 was severely arrested under salinity stress, more so than that of JS-7 (Fig. 4-1A), indicating that changes in the optimal pH for cell wall loosening are important for root-growth regulation under salinity stress. In summary, salinity stress reduced cell wall extensibility in both cultivars. However, apoplastic acidification and changes in expansin expression differently regulated cell wall extensibility in the two cultivars. As a result, under salinity stress, apoplastic acidification further stiffened the root cell walls and slowed root growth in YL-15, whereas, in JS-7, it loosened the root cell walls and mitigated the inhibition of root growth triggered by Na^+.

4.5.3 Expansin expression under salinity stress

The differences in pH-dependent cell wall extensibility between the sensitive and tolerant cultivars were associated with the differential expression of α-expansins (Fig. 4-5). Expression levels of *TaEXPA5* and *TaEXPA8* were higher in JS-7 than in YL-15 under salinity stress; in JS-7, expansin caused cell wall loosening in the acidified apoplast. Under normal condition, lower *TaEXPA3*, *TaEXPA7* and *TaEXPB10* expressions in roots of JS-7 than YL-15, which was closely related to the differences in the optimal pH for cell wall loosening between two cultivars. The different expression patterns of expansin under salinity stress indicates the elevated expressions of *TaEXPA5* and *TaEXPA8* in JS-7 may contribute to cell wall loosening salinity stress. The elevated expression of *TaEXPA5* (Fig. 5) and cell wall loosening at pH 6.0 (Fig. 4-4A, B, C), in both cultivars under the NaCl treatment, indicate that *TaEXPA5* may

contribute to the cell wall loosening at pH 6.0. Our co-expression analysis showed the *TaEXPA5* regulated the number of roots, whereas *TaEXPA8* was highly associated with cell lengthening under various stresses. *TaEXPA8* is reported to be closely related to cold tolerance in wheat (Zhang et al. 2018), and the overexpression of *TaEXPA8* improves cold tolerance in transgenic *Arabidopsis* (Peng et al. 2019). These results suggest the importance of *TaEXPA8* in enhancing root growth under various abiotic stresses. A recent study has shown that *AtEXPA1* overexpression alters the optimal pH for cell wall loosening in *Arabidopsis* (Samalova et al. 2020). Therefore, the differential expansin expression between the two wheat cultivars, both under normal conditions and salinity stress, suggests that cell wall loosening at pH 5.0 and pH 6.0 is caused by different expansin genes: *TaEXPA8* may induce cell wall loosening at pH 5.0, whereas *TaEXPA5* may induce it at pH 6.0.

Expansin genes expressed in wheat coleoptiles induce cell wall loosening under low apoplastic pH (pH of 4.0–4.5; Gao et al. 2008). Further, overexpression of the expansin genes specifically expressed in wheat coleoptiles (such as *TaEXPB23* and *TaEXPA2*) can enhance root growth under salinity stress (Han et al. 2012; Zhao et al. 2012). These results are consistent with our findings. Thus, I conclude that, under the apoplastic acidification caused by salinity stress, the salt-tolerant cultivar elevates its expression of the expansin genes (such as *TaEXPA5* and *TaEXPA8*), which may shift the optimal pH for cell wall loosening from 6.0 to 5.0. This change in the optimal pH for cell wall loosening enables the salinity-tolerant cultivar to maintain a relative higher root growth than the sensitive cultivar.

Further, the root expansin extract contains various kinds of expansins, and they are hard to be separated because they have similar molecular weight and isoelectric point. Based on the RNA-seq data from GenBank (accession SRP062745), certain α-expansin, β-expansin, and expansin-like A (*TaEXPLA*) genes are highly expressed only under salinity stress. These stress-specific expansin genes may also play a critical role in responses to salinity stress. The α-

expansin, β-expansin, and expansin-like A genes related to salinity stress need to be further studied.

4.5.4 Characterization of cell wall expansins

Drought stress does not change the susceptibility of root cell walls to exogenous expansin in wheat (Zhao et al. 2011). Interestingly, at pH 5.0, the expansin extracted from JS-7 80 mM induced the highest root cell wall η_N extensibility in YL-15 80 mM roots, whereas that from YL-15 0 mM induced the lowest extensibility in YL-15 0 mM roots (Fig. 4-4A, B). This indicates that salinity stress alters cell wall susceptibility to exogenous expansin in YL-15. Changes in cell wall susceptibility to salinity stress are related to cell wall structure and composition. Salinity stress alters cell wall structure, resulting in a stiff and mesh-like network, suggesting an increase in cellulose-xyloglucan conjunctions (Koyro 1997). The increased xyloglucan then strengthens the linkages between cellulose molecules; this process reduced the growth rate in coffee (*Coffea arabica*) leaf cells under salinity stress (De Lima et al. 2014). Thus, changes in cell wall susceptibility to expansins may be closely related to changes in cell wall composition. Further research on changes in cell wall properties under salinity stress is necessary to elucidate cell wall susceptibility to expansins.

In conclusion, the apoplastic acidification of apical roots in response to salinity stress stiffens the cell walls and inhibits root growth in the salt-sensitive wheat cultivar. However, under salinity stress, elevated *TaEXPA8* expression may mitigate cell wall stiffness and enhances root growth in the salt-tolerant cultivar, via apoplastic acidification.

5. General discussion

5.1 Physio-biochemical properties of root cell wall

As the outermost layer that constrains the cell growth, the cell wall is composed of relatively stiff cellulose microfibrils embedded in a hydrated matrix of pectin and hemicellulose (Cosgrove, 2018). Within the cell wall, pectin, ions, and proteins maintain a stable microenvironment. In all cultivars used in this study except for JS-7, salt stress decreased pectin content in the first zone (Fig. 2-2). In sensitive cultivars, hemicellulose I and II concentrations were significantly higher in the first and second zones than in tolerant cultivars (Fig. 2-2). All cultivars in both zones showed an increase in cellulose content; this increment was pronounced in sensitive cultivars (Fig. 2-2). The reduced uronic acid content in pectin was observed in sensitive cultivars compared with tolerant cultivars in the first zone, while a higher content of uronic acid was noted in hemicellulose in the second zone (Fig. 2-3). Salinity stress also altered the apoplastic pH (Fig. 4-2) and expansin expression (Fig. 4-5). For both cultivars, the apoplastic pH was ~6.3 under 0 mM NaCl condition, whereas the pH was ~5.3 at under 80 mM treatment, indicating that long-term salinity stress significantly acidified the apical-root apoplast. Gene expressions of *TaEXPA3*, *TaEXPA6, TaEXPB1* and *TaEXPB10* were reduced in both cultivars under salinity stress (Fig. 4-5). However, those of *TaEXPA5* and *TaEXPA8* in tolerant cultivars increased under salinity. The changes in physio-biochemical properties of cell wall under salinity were closely correlated with the CEC and extension of the cell walls.

5.2 Interactions of wall related parameters

The uronic acid-rich polysaccharides are the main ion-binding sites in the cell walls under physiological conditions (Meychik and Yermakov 2000). The CEC of the total cell wall was positively correlated with the relative uronic acid content in pectin in the root first zone (Table 1-1), indicating that the uronic acid in pectin contributes to the interaction between Na^+ and the

cell wall. In the second zone, the close correlation between the CEC of the root cell walls, relative uronic acid content in pectin, and hemicellulose I indicates that the development of cell wall, the ion-binding sites of both pectin, and hemicellulose I may be involved in the enhancement of the CEC in the cell wall (Table 2-2).

The acidic linkages in cellulose and hemicellulose are the main load-bearings in cell wall (Cosgrove 2018). Cell wall elasticity was positively correlated with the relative pectin and hemicellulose I contents and negatively correlated with the relative cellulose content, indicating that high levels of pectin and low levels of cellulose significantly increase the cell wall extensibility of root tips (Table 3-3). The uronic acid content in cellulose increased dramatically in the first and second zones in all cultivars with NaCl treatments, except XC-31 in the first zone (Fig. 2-3). A high uronic acid content in cellulose and hemicellulose indicates that they form more acidic linkages under salinity stress than control condition. In the salt-sensitive cultivars, the magnitudes of increase in uronic acid in hemicellulose and cellulose were pronounced than in the tolerant ones (Fig. 2-3). This result indicates the salt-sensitive cultivars form more acidic linkages than tolerant ones, leading to the significant loss of elastic properties (Fig. 3-2). Therefore, the high content of pectin and hemicellulose I in salt-tolerant cultivars maintained the higher extensibility under salt stress compared with the sensitive ones, whereas the increase of cellulose and uronic acid in hemicellulose and cellulose in sensitive cultivars leads to the decrease of extensibility, which further inhibits the root growth under salinity stress and reduces salt tolerance.

Under salinity stress, sodium ions compete for the ion binding sites in pectin. When Na^+ binds to pectin, H^+ is released to the cell wall. Therefore, under salinity stress, the apoplastic pH decreased (Fig. 4-2E). There were significant differences in cation exchange capacity between JS-7 and YL-15 (Fig. 2-4). However, two cultivars showed no obvious difference in apoplastic pH (Fig. 4-2E). This indicates the cation exchange capacity is not the only factor

that causes the acidification of cell wall. The Na^+ extrude ability may also induce the decrease of apoplastic pH: when wheat roots sense Na^+, the proton pumps such as ATPase are activated (Byrt et al., 2018). Subsequently, protons in the cytosol are pumped across the membrane. While protons return into the cell cytosol due to the potential difference between the membrane, *SOS1*, a Na^+/H^+ exchange protein, is activated to extrude excess Na^+ from the cells (Zhu et al. 2016). Through this process, the apoplastic pH is decreased.

Expansion of the tolerant cultivar was more beneficial at low pH than the sensitive one, as expansin loosened cell walls at the same pH (Fig. 4-4). The results of qPCR showed salinity stress increased alpha-expansin expression (*TaEXPA5* and *TaEXPA8*) in salt-tolerant cultivar, which induced the cell wall loosening under low and high apoplastic pH (Fig. 4-5). In salt-tolerant cultivars, *TaEXPA5* and *TaEXPA8* expression was elevated under salinity stress, enhancing cell wall relaxation at low apoplastic pH. The relaxation of cell wall alleviates the growth rate inhibition associated with decreased turgor pressure. In contrast, sensitive cells exhibited the opposite effect of stiffening the cell walls, which inhibited cell expansion further. In chapter 4, the cell wall showed different susceptivity to expansins. YL-15 showed the higher susceptibility to the expansins (Fig. 4-4A, B), along with the higher uronic acid content in hemicellulose and cellulose than JS-7 (Fig. 2-3). These results indicate the susceptibility of cell walls to expansins is closely related to the changes in wall composition, especially the amount of uronic acid. A high amount of uronic acid in hemicellulose and cellulose increases the susceptibility of cell walls to expansins.

5.3 Characterization of root cell wall in wheat cultivars

In *Suaeda altissima* (Meychik et al. 2006) and barley (Flowers and Hajibagheri 2001), the CEC of the cell walls in the mature zone is highly associated with salt tolerance. In the present study, salt treatment significantly decreased the CEC of the cell wall in all the wheat cultivars

(Fig. 2-4). The decreased CEC under saline condition suggests that Na^+ affects the cell wall components that are involved in cation binding (Fig. 2-3 and Fig. 2-4). The CEC of the root cell walls in tolerant cultivars was significantly lower than the sensitive cultivars under the 0 mM and 80 mM treatments. The high CEC under the saline condition and the increase in Na^+ concentration in the tolerant cultivars (Fig. 2-4) imply that the tolerant cultivars bind more Na^+ in the root cell wall than the sensitive cultivars.

Salt stress alters cell wall structure, resulting in a rigid, mesh-like network, suggesting an increase in hemicellulose-cellulose conjunctions (Koyro 1997). As a result of increased hemicellulose, cellulose microfibrils are strengthened, thereby reducing cell growth in coffee (*Coffea arabica*) leaves under salinity stress (De Lima et al. 2014). Elasticity of the root cell wall, indicated by E_0, significantly decreased in the salt-sensitive cultivars, whereas the E_0 in the salt-tolerant cultivars was maintained at the same level as that in the non-saline condition (Table 3-2). Root extension and the differences among cultivars were largely dependent on elastic extension, which accounts for one-half to two-thirds of the total extension (Fig. 3-2). Viscosity, indicated by η_0, and the plastic extension of the root cell walls did not change across the treatments and cultivars (Table 3-2). The significant decrease in cell wall elasticity in the root elongation region was one of the factors that depressed root growth in salt-sensitive cultivars under the saline condition. The well-maintained elasticity of salt-tolerant cultivars alleviated the depression of root growth by NaCl.

In response to saline treatment, salt tolerant plants had faster root growth than sensitive plants, indicating that tolerant plants have superior cell elongation ability under salinity stress. The roots of both cultivars showed a decrease in pH due to salinity stress. In contrast, under salinity stress, apoplastic acidification induced cell wall loosening in tolerant but not in sensitive cultivar. The root growth of sensitive cultivar was severely arrested under salinity

stress, more so than that of tolerant cultivar (Fig. 4-1A), indicating that changes in the optimal pH for cell wall loosening are important for root-growth regulation under salinity stress.

5.4 Functions of root cell wall in salinity tolerance in wheat

High CEC in cell wall means more sodium ions can be stored in the root wall, which is useful for reducing cytosolic Na^+ accumulation and improving salt tolerance. Previous study shows that sodium ions can disrupt the structure of the cell wall (Feng et al. 2018), while high amount of uronic acid helps to maintain the stabilization of cell wall and contributes to a hydrated environment around cell membranes, supporting a homeostatic microenvironment for cell wall bound hydrolases and ion transportation. High pectin content and cation exchange capacity in the cell wall of are useful for maintaining tolerance under salt stress conditions.

Maintaining root growth is a very important feature of wheat growth, which is highly related to salt tolerance (Mujeeb-Kazi et al., 2019). In root cells, cell elongation depends on turgor pressure and cell wall extensibility. Excessive salt causes the hyper-ionic and hyper-osmotic stress to plant cells (Shabala 2017), which decreases the turgor pressure of cells. The cell wall extensibility becomes a critical factor affecting the growth rate of cells in this scenario. In salt-tolerant cultivars, changes in chemical composition of the cell wall correspond with the well-maintained elasticity, which alleviated the depression of root growth by NaCl (Fig. 3-2). Further, under salinity stress with lowered apoplastic pH (Fig. 4-2), expansins in the tolerant cultivar resulted in the maintenance of cell wall extensibility, whereas those in the sensitive cultivars had no such activity. High cell wall extensibility is important for maintaining root growth and tolerance under salt stress conditions.

The overall result reveals that salinity stress affects cell wall compositions, apoplastic pH and expansin expression. These changes subsequently affect cation exchange capacity and cell wall extensibility of wheat roots. Under salinity stress, wheat needs to coordinate the cell wall

properties in harmony: increasing pectin, hemicellulose I and uronic acid contents in pectin, while maintaining low hemicellulose II and cellulose contents; tuning the pH-dependent extensibility to accommodate the apoplastic acidification. This study provides insight into the relationship between physical, chemical, and biological properties of root cell walls, which can be used to enhance plant growth under salinity stress.

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References

Al-Hakimi AMA, Hamada AM (2001) Counteraction of salinity stress on wheat plants by grain soaking in ascorbic acid, thiamin or sodium salicylate. Biol Plant 44:253–261. https://doi.org/10.1023/A:1010255526903

Al-Yasi H, Attia H, Alamer K, et al (2020) Impact of drought on growth, photosynthesis, osmotic adjustment, and cell wall elasticity in Damask rose. Plant Physiol Biochem 150:133–139. https://doi.org/10.1016/j.plaphy.2020.02.038

An P, Inanaga S, Cohen Y, et al (2002) Salt tolerance in two soybean cultivars. J Plant Nutr 25:407–423. https://doi.org/10.1081/PLN-120003373

An P, Li X, Zheng Y, et al (2014a) Effects of NaCl on root growth and cell wall composition of two soya bean cultivars with contrasting salt tolerance. J Agron Crop Sci 200:212–218. https://doi.org/10.1111/jac.12060

An P, Li X, Zheng Y, et al (2014b) Calcium effects on root cell wall composition and ion contents in two soybean cultivars under salinity stress. Can J Plant Sci 94:733–740. https://doi.org/10.4141/CJPS2013-291

Appels R, Eversole K, Feuillet C, et al (2018) Shifting the limits in wheat research and breeding using a fully annotated reference genome. Science 361:. https://doi.org/10.1126/science.aar7191

Asgari HR, Cornelis W, Van Damme P (2012) Salt stress effect on wheat (*Triticum aestivum* L.) growth and leaf ion concentrations. Int J Plant Prod 6:195–208. https://doi.org/10.22069/ijpp.2012.775

Aslan D, Zencırcı N, Etöz M, et al (2016) Bread wheat responds salt stress better than einkorn wheat does during germination. Turk J Agric For 40:783–794. https://doi.org/10.3906/tar-1604-59

Barbez E, Dünser K, Gaidora A, et al (2017) Auxin steers root cell expansion via apoplastic pH regulation in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A 114:E4884–E4893. https://doi.org/10.1073/pnas.1613499114

Beemster GTS, Baskin TI (1998) Analysis of cell division and elongation underlying the developmental acceleration of root growth in *Arabidopsis thaliana*. Plant Physiol 116:1515–1526. https://doi.org/10.1104/pp.116.4.1515

Blumenkrantz N, Asboe-Hansen G (1973) New method for quantitative determination of uronic acids. Anal Biochem 54:484–489. https://doi.org/10.1016/0003-2697(73)90377-1

Boudaoud A (2010) An introduction to the mechanics of morphogenesis for plant biologists. Trends Plant Sci 15:353–360. https://doi.org/10.1016/j.tplants.2010.04.002

Bustin SA, Benes V, Garson JA, et al (2009) The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622. https://doi.org/10.1373/clinchem.2008.112797

Byrt CS, Munns R, Burton RA, et al (2018) Root cell wall solutions for crop plants in saline soils. Plant Science

Chen Y, Han Y, Kong X, et al (2017) Ectopic expression of wheat expansin gene *TaEXPA2* improved the salt tolerance of transgenic tobacco by regulating Na^+/K^+ and antioxidant competence. Physiol Plant 159:161–177. https://doi.org/10.1111/ppl.12492

Colzi I, Arnetoli M, Gallo A, et al (2012) Copper tolerance strategies involving the root cell wall pectins in *Silene paradoxa* L. Environ Exp Bot 78:91–98. https://doi.org/10.1016/j.envexpbot.2011.12.028

Corrêa-Ferreira ML, Viudes EB, de Magalhães PM, et al (2019) Changes in the composition and structure of cell wall polysaccharides from *Artemisia annua* in response to salt stress. Carbohydr Res 483:107753. https://doi.org/10.1016/j.carres.2019.107753

Cosgrove DJ (2018) Diffuse growth of plant cell walls. Plant Physiol 176:16–27. https://doi.org/10.1104/pp.17.01541

Cosgrove DJ (2000) Loosening of plant cell walls by expansins. Nature 407:321–326. https://doi.org/10.1038/35030000

Cosgrove DJ (2005) Growth of the plant cell wall. Nat Rev Mol Cell Biol 6:850–861. https://doi.org/10.1038/nrm1746

Crooke WM (1964) The measurement of the cation-exchange capacity of plant roots. Plant Soil 21:43–49. https://doi.org/10.1007/BF01373871

Cuin TA, Tian Y, Betts SA, et al (2009) Ionic relations and osmotic adjustment in durum and bread wheat under saline conditions. Funct Plant Biol 36:1110–1119. https://doi.org/10.1071/FP09051

Dang X, Chen B, Liu F, et al (2020) Auxin signaling-mediated apoplastic pH modification functions in petal conical cell shaping. Cell Rep 30:3904-3916.e3. https://doi.org/10.1016/j.celrep.2020.02.087

Davis TA, Volesky B, Mucci A (2003) A review of the biochemistry of heavy metal biosorption by brown algae. Pergamon

De Lima RB, Dos Santos TB, Vieira LGE, et al (2014) Salt stress alters the cell wall polysaccharides and anatomy of coffee (*Coffea arabica* L.) leaf cells. Carbohydr Polym 112:686–694. https://doi.org/10.1016/j.carbpol.2014.06.042

Deinlein U, Stephan AB, Horie T, et al (2014) Plant salt-tolerance mechanisms. Trends Plant Sci 19:371–379. https://doi.org/10.1016/j.tplants.2014.02.001

Duan L, Dietrich D, Ng CH, et al (2013) Endodermal ABA Signaling Promotes Lateral Root Quiescence during Salt Stress in Arabidopsis Seedlings. Plant Cell 25:324–341. https://doi.org/10.1105/tpc.112.107227

Dubois M, Gilles K, Hamilton JK, et al (1951) A colorimetric method for the determination of sugars. Nature 168:167–167. https://doi.org/10.1038/168167a0

Fan L, Linker R, Gepstein S, et al (2006) Progressive inhibition by water deficit of cell wall extensibility and growth along the elongation zone of maize roots is related to increased lignin metabolism and progressive stelar accumulation of wall phenolics. Plant Physiol 140:603–612. https://doi.org/10.1104/pp.105.073130

Fang C, Li K, Wu Y, et al (2019) *OsTSD2*-mediated cell wall modification affects ion homeostasis and salt tolerance. Plant Cell Environ 42:1503–1512. https://doi.org/10.1111/pce.13499

FAO (2015) FAO Statistical Pocketbook 2015

Felle HH, Hanstein S (2002) The apoplastic pH of the substomatal cavity of *Vicia faba* leaves and its regulation responding to different stress factors. J Exp Bot 53:73–82. https://doi.org/10.1093/jexbot/53.366.73

Felle HH, Herrmann A, Hückelhoven R, Kogel KH (2005) Root-to-shoot signalling: Apoplastic alkalinization, a general stress response and defence factor in barley (*Hordeum vulgare*). Protoplasma 227:17–24. https://doi.org/10.1007/s00709-005-0131-5

Feng W, Kita D, Peaucelle A, et al (2018) The FERONIA receptor kinase maintains cell-wall integrity during salt stress through Ca^{2+} Signaling. Curr Biol 28:666-675.e5. https://doi.org/10.1016/j.cub.2018.01.023

Flowers TJ, Hajibagheri MA (2001) Salinity tolerance in *Hordeum vulgare*: Ion concentrations in root cells of cultivars differing in salt tolerance. Plant Soil 231:1–9. https://doi.org/10.1023/A:1010372213938

Fry SC (2011) Plant cell walls. From chemistry to biology. Ann Bot 108:viii–ix. https://doi.org/10.1093/aob/mcr128

Fry SC (2004) Primary cell wall metabolism: Tracking the careers of wall polymers in living plant cells. New Phytol 161:641–675. https://doi.org/10.1111/j.1469-8137.2004.00980.x

Gao D, Knight MR, Trewavas AJ, et al (2004) Self-reporting Arabidopsis expressing pH and [Ca^{2+}] indicators unveil ion dynamics in the cytoplasm and in the apoplast under abiotic stress. Plant Physiol 134:898–908. https://doi.org/10.1104/pp.103.032508

Gao Q, Zhao M, Li F, et al (2008) Expansins and coleoptile elongation in wheat. Protoplasma 233:73–81. https://doi.org/10.1007/s00709-008-0303-1

Geilfus CM (2017) The pH of the apoplast: Dynamic factor with functional impact under stress. Mol Plant 10:1371–1386. https://doi.org/10.1016/j.molp.2017.09.018

Geilfus C-M, Mithöfer A, Ludwig-Müller J, et al (2015) Chloride-inducible transient apoplastic alkalinizations induce stomata closure by controlling abscisic acid distribution between leaf apoplast and guard cells in salt-stressed *Vicia faba*. New Phytol 208:803–816. https://doi.org/10.1111/nph.13507

Geilfus CM, Tenhaken R, Carpentier SC (2017) Transient alkalinization of the leaf apoplast stiffens the cell wall during onset of chloride salinity in corn leaves. J Biol Chem 292:18800–18813. https://doi.org/10.1074/jbc.M117.799866

Geilfus C-MM, MÜHLING KH (2012) Transient alkalinization in the leaf apoplast of *Vicia faba* L. depends on NaCl stress intensity: an *in situ* ratio imaging study. Plant Cell Environ 35:578–587. https://doi.org/10.1111/j.1365-3040.2011.02437.x

Glazowska S, Baldwin L, Mravec J, et al (2018) The impact of silicon on cell wall composition and enzymatic saccharification of *Brachypodium distachyon*. Biotechnol Biofuels 11:171. https://doi.org/10.1186/s13068-018-1166-0

Grant GT, Morris ER, Rees DA, et al (1973) Biological interactions between polysaccharides and divalent cations: The egg-box model. FEBS Lett 32:195–198. https://doi.org/10.1016/0014-5793(73)80770-7

Han J, Burgess K (2010) Fluorescent indicators for intracellular pH. Chem Rev 110:2709–2728. https://doi.org/10.1021/cr900249z

Han YY, Li AX, Li F, et al (2012) Characterization of a wheat (Triticum aestivum L.) expansin gene, *TaEXPB23*, involved in the abiotic stress response and phytohormone regulation. Plant Physiol Biochem 54:49–58. https://doi.org/10.1016/j.plaphy.2012.02.007

Han Z, Liu Y, Deng X, et al (2019) Genome-wide identification and expression analysis of expansin gene family in common wheat (*Triticum aestivum* L.). BMC Genomics 20:101. https://doi.org/10.1186/s12864-019-5455-1

Harrison EP, McQueen-Mason SJ, Manning K (2001) Expression of six expansin genes in relation to extension activity in developing strawberry fruit. J Exp Bot 52:1437–1446. https://doi.org/10.1093/jexbot/52.360.1437

Hassani-Pak K (2017) KnetMiner - An integrated data platform for gene mining and biological knowledge discovery. PhD Thesis

Hattori T, Inanaga S, Tanimoto E, et al (2003) Silicon-induced changes in viscoelastic properties of sorghum root cell walls. Plant Cell Physiol 44:743–749. https://doi.org/10.1093/pcp/pcg090

Hongo S, Sato K, Yokoyama R, Nishitani K (2012) Demethylesterification of the primary wall by PECTIN METHYLESTERASE35 provides mechanical support to the *Arabidopsis* stem. Plant Cell 24:2624–2634. https://doi.org/10.1105/tpc.112.099325

Hossain MT, Mori R, Soga K, et al (2002) Growth promotion and an increase in cell wall extensibility by silicon in rice and some other *Poaceae* seedlings. J Plant Res 115:23–27. https://doi.org/10.1007/s102650200004

Hossain MT, Soga K, Wakabayashi K, Hoson T (2015) Effects of lead toxicity on growth and cell wall extensibility in rice seedlings. Bangladesh J Bot 44:333–336. https://doi.org/10.3329/bjb.v44i2.38526

Inukai Y, Sakamoto T, Morinaka Y, et al (2012) Root growth inhibiting, a rice endo-1, 4-β-D-glucanase, regulates cell wall loosening and is essential for root elongation. J Plant Growth Regul 31:373–381. https://doi.org/10.1007/s00344-011-9247-3

Iraki NM, Singh N, Bressan RA, Carpita NC (1989) Cell walls of tobacco cells and changes in composition associated with reduced growth upon adaptation to water and saline stress. Plant Physiol 91:48–53. https://doi.org/10.1104/pp.91.1.48

Jian LY, Ya YL, Yue JZ, et al (2008) Cell wall polysaccharides are specifically involved in the exclusion of aluminum from the rice root apex. Plant Physiol 146:602–611. https://doi.org/10.1104/pp.107.111989

Jixiang Lin (2012) Salinity-alkalinity tolerance in wheat: Seed germination, early seedling growth, ion relations and solute accumulation. Afr J Agric Reseearch 7:467–474. https://doi.org/10.5897/ajar11.1417

Jung NU, Giarola V, Chen P, et al (2019) *Craterostigma plantagineum* cell wall composition is remodelled during desiccation and the glycine-rich protein CpGRP1 interacts with pectins through clustered arginines. Plant J 100:661–676. https://doi.org/10.1111/tpj.14479

Kaku T, Tabuchi A, Wakabayashi K, et al (2002) Action of xyloglucan hydrolase within the native cell wall architecture and its effect on cell wall extensibility in azuki bean epicotyls. Plant Cell Physiol 43:21–26. https://doi.org/10.1093/pcp/pcf004

Koyama H, Toda T, Hara T (2001) Brief exposure to low-pH stress causes irreversible damage to the growing root in *Arabidopsis thaliana*: pectin–Ca interaction may play an important role in proton rhizotoxicity. J Exp Bot 52:361–368. https://doi.org/10.1093/jexbot/52.355.361

Koyro H-W (1997) Ultrastructural and physiological changes in root cells of sorghum plants (*Sorghum bicolor* x *S. sudanensis* cv. Sweet Sioux) induced by NaCl

Leucci MR, Lenucci MS, Piro G, Dalessandro G (2008) Water stress and cell wall polysaccharides in the apical root zone of wheat cultivars varying in drought tolerance. J Plant Physiol 165:1168–1180. https://doi.org/10.1016/j.jplph.2007.09.006

Li T, Tao Q, Shohag MJI, et al (2015) Root cell wall polysaccharides are involved in cadmium hyperaccumulation in *Sedum alfredii*. Plant Soil 389:387–399. https://doi.org/10.1007/s11104-014-2367-3

Li W, Guan Q, Wang ZY, et al (2013) A bi-functional xyloglucan galactosyltransferase is an indispensable salt stress tolerance determinant in *Arabidopsis*. Mol Plant 6:1344–1354. https://doi.org/10.1093/mp/sst062

Lin Z, Ni Z, Zhang Y, et al (2005) Isolation and characterization of 18 genes encoding α- and β-expansins in wheat (*Triticum aestivum* L.). Mol Genet Genomics 274:548–556. https://doi.org/10.1007/s00438-005-0029-0

Lutts S, Qin P, Han RM (2016) Salinity influences biosorption of heavy metals by the roots of the halophyte plant species *Kosteletzkya pentacarpos*. Ecol Eng 95:682–689. https://doi.org/10.1016/j.ecoleng.2016.06.009

Ma JF, Shen R, Nagao S, Tanimoto E (2004) Aluminum targets elongating cells by reducing cell wall extensibility in wheat roots. Plant Cell Physiol 45:583–589. https://doi.org/10.1093/pcp/pch060

Mahajan MM, Goyal E, Singh AK, et al (2020) Shedding light on response of *Triticum aestivum* cv. Kharchia Local roots to long-term salinity stress through transcriptome profiling. Plant Growth Regul 90:369–381. https://doi.org/10.1007/s10725-019-00565-4

Majda M, Robert S (2018) The role of auxin in cell wall expansion. Int J Mol Sci 19:951. https://doi.org/10.3390/ijms19040951

McKenna BA, Kopittke PM, Wehr JB, et al (2010) Metal ion effects on hydraulic conductivity of bacterial cellulose-pectin composites used as plant cell wall analogs. Physiol Plant 138:205–214. https://doi.org/10.1111/j.1399-3054.2009.01306.x

McQueen-Mason S, Durachko DM, Cosgrove DJ (1992) Two endogenous proteins that induce cell wall extension in plants. Plant Cell 4:1425–1433. https://doi.org/10.1105/tpc.4.11.1425

Mellerowicz EJ, Sundberg B (2008) Wood cell walls: biosynthesis, developmental dynamics and their implications for wood properties. Elsevier Current Trends

Meychik N, Nikolaeva Y, Kushunina M, Yermakov I (2014) Are the carboxyl groups of pectin polymers the only metal-binding sites in plant cell walls? Plant Soil 381:25–34. https://doi.org/10.1007/s11104-014-2111-z

Meychik NR, Nikolaeva YI, Yermakov IP (2006) Ion-exchange properties of cell walls of *Spinacia oleracea* L. roots under different environmental salt conditions. Biochem Mosc 71:781–789. https://doi.org/10.1134/S000629790607011X

Meychik NR, Yermakov IP (2000) A new approach to the investigation on the tonogenic groups of root cell walls. Support Roots Trees Woody Plants Form Funct Physiol 217:419–426. https://doi.org/10.1007/978-94-017-3469-1_41

Meychik NR, Yermakov IP, Khonarmand SD, Nikolaeva YI (2010) Ion-exchange properties of cell walls in chickpea cultivars with different sensitivities to salinity. Russ J Plant Physiol 57:620–630. https://doi.org/10.1134/S1021443710050043

Miedes E, Zarra I, Hoson T, et al (2011) Xyloglucan endotransglucosylase and cell wall extensibility. J Plant Physiol 168:196–203. https://doi.org/10.1016/j.jplph.2010.06.029

Mujeeb-Kazi A, Munns R, Rasheed A, et al (2019) Breeding strategies for structuring salinity tolerance in wheat. In: Advances in Agronomy. Academic Press Inc., pp 121–187

Munns R, James RA, Läuchli A (2006) Approaches to increasing the salt tolerance of wheat and other cereals. In: Journal of Experimental Botany. pp 1025–1043

Munns R, Passioura JB, Colmer TD, Byrt CS (2020) Osmotic adjustment and energy limitations to plant growth in saline soil. New Phytol 225:1091–1096. https://doi.org/10.1111/nph.15862

Ogawa A, Yamauchi A (2006) Root osmotic adjustment under osmotic stress in maize seedlings 1. Transient change of growth and water relations in roots in response to osmotic stress. Plant Prod Sci 9:27–38. https://doi.org/10.1626/pps.9.27

O'Neill MA, Ishii T, Albersheim P, Darvill AG (2004) RHAMNOGALACTURONAN II: Structure and function of a borate cross-linked cell wall pectic polysaccharide. Annu Rev Plant Biol 55:109–139. https://doi.org/10.1146/annurev.arplant.55.031903.141750

Pacifici E, Di Mambro R, Dello Ioio R, et al (2018) Acidic cell elongation drives cell differentiation in the *Arabidopsis* root. EMBO J 37:. https://doi.org/10.15252/embj.201899134

Palin R, Geitmann A (2012) The role of pectin in plant morphogenesis. BioSystems 109:397–402. https://doi.org/10.1016/j.biosystems.2012.04.006

Park YB, Cosgrove DJ (2012) Changes in cell wall biomechanical properties in the xyloglucan-deficient *xxt1*/*xxt2* mutant of *Arabidopsis*. Plant Physiol 158:465–475. https://doi.org/10.1104/pp.111.189779

Pelloux J, Rustérucci C, Mellerowicz EJ (2007) New insights into pectin methylesterase structure and function. Trends Plant Sci 12:267–277. https://doi.org/10.1016/j.tplants.2007.04.001

Peng LN, Xu YQ, Wang X, et al (2019) Overexpression of paralogues of the wheat expansin gene *TaEXPA8* improves low-temperature tolerance in Arabidopsis. Plant Biol 21:1119–1131. https://doi.org/10.1111/plb.13018

Podgórska A, Burian M, Gieczewska K, et al (2017) Altered cell wall plasticity can restrict plant growth under ammonium nutrition. Front Plant Sci 8:. https://doi.org/10.3389/fpls.2017.01344

Qiu ZB, Guo JL, Zhu AJ, et al (2014) Exogenous jasmonic acid can enhance tolerance of wheat seedlings to salt stress. Ecotoxicol Environ Saf 104:202–208. https://doi.org/10.1016/j.ecoenv.2014.03.014

Rahnama A, Munns R, Poustini K, Watt M (2011) A screening method to identify genetic variation in root growth response to a salinity gradient. J Exp Bot 62:69–77. https://doi.org/10.1093/jxb/erq359

Rayle DL, Cleland RE (1992) The acid growth theory of auxin-induced cell elongation is alive and well. Plant Physiol 99:1271–1274. https://doi.org/10.1104/pp.99.4.1271

Renault S, Zwiazek JJ (1997) Cell wall composition and elasticity of dormant and growing white spruce (*Picea glauca*) seedlings. Physiol Plant 101:323–327. https://doi.org/10.1111/j.1399-3054.1997.tb01003.x

rong Zhao M, Li F, Fang Y, et al (2011) Expansin-regulated cell elongation is involved in the drought tolerance in wheat. Protoplasma 248:313–323. https://doi.org/10.1007/s00709-010-0172-2

rong Zhao M, yang Han Y, nan Feng Y, et al (2012) Expansins are involved in cell growth mediated by abscisic acid and indole-3-acetic acid under drought stress in wheat. Plant Cell Rep 31:671–685. https://doi.org/10.1007/s00299-011-1185-9

Rygol J, Zimmermann U (1990) Radial and axial turgor pressure measurements in individual root cells of *Mesembryanthemum crystallinum* grown under various saline conditions. Plant Cell Environ 13:15–26. https://doi.org/10.1111/j.1365-3040.1990.tb01295.x

Sadat Noori SA, McNeilly T (2000) Assessment of variability in salt tolerance based on seedling growth in Triticum durum Desf. Genet Resour Crop Evol 47:285–291. https://doi.org/10.1023/A:1008749312148

Safari M, Ghanati F, Safarnejad MR, Chashmi NA (2018) The contribution of cell wall composition in the expansion of *Camellia sinensis* seedlings roots in response to aluminum. Planta 247:381–392. https://doi.org/10.1007/s00425-017-2792-7

Sakurai N (1991) Cell wall functions in growth and development -a physical and chemical point of view. Bot Mag Tokyo 104:235–251. https://doi.org/10.1007/BF02489456

Sakurai N, Tanaka S, Kuraishi S (1987) Changes in wall polysaccharides of squash (*Cucurbita maxima* Duch.) hypocotyls under water stress condition: I. Wall sugar composition and growth as affected by water stress. Plant Cell Physiol 28:1051–1058. https://doi.org/10.1093/oxfordjournals.pcp.a077385

Samalova M, Elsayad K, Melnikava A, et al (2020) Expansin-controlled cell wall stiffness regulates root growth in *Arabidopsis*. bioRxiv 2020.06.25.170969. https://doi.org/10.1101/2020.06.25.170969

Scheller HV, Ulvskov P (2010) Hemicelluloses. Annu Rev Plant Biol 61:263–289. https://doi.org/10.1146/annurev-arplant-042809-112315

Shabala S (2017) Plant stress physiology (2nd Edition). Cabi

Shigeyama T, Watanabe A, Tokuchi K, et al (2016) α-Xylosidase plays essential roles in xyloglucan remodelling, maintenance of cell wall integrity, and seed germination in *Arabidopsis thaliana*. J Exp Bot 67:5615–5629. https://doi.org/10.1093/jxb/erw321

Siedlecka A, Wiklund S, Péronne MA, et al (2008) Pectin methyl esterase inhibits intrusive and symplastic cell growth in developing wood cells of *Populus*. Plant Physiol 146:554–565. https://doi.org/10.1104/pp.107.111963

Singh A, Prasad R (2009) Salt stress effects growth and cell wall bound enzymes in *Arachis hypogaea* L. seedlings. Int J Integr Biol 7:117–123

Solomon M, Ariel R, Hodson MJ, et al (1987) Ion absorption and allocation of carbon resources in excised pea roots grown in liquid medium in absence or presence of NaCl. Ann Bot 59:387–398. https://doi.org/10.1093/oxfordjournals.aob.a087328

Szatanik-Kloc A, Szerement J, Józefaciuk G (2017) The role of cell walls and pectins in cation exchange and surface area of plant roots. J Plant Physiol 215:85–90. https://doi.org/10.1016/j.jplph.2017.05.017

Tabuchi A, Matsumoto H (2001) Changes in cell-wall properties of wheat (*Triticum aestivum*) roots during aluminum-induced growth inhibition. Physiol Plant 112:353–358. https://doi.org/10.1034/j.1399-3054.2001.1120308.x

Tan L, Eberhard S, Pattathil S, et al (2013) An *Arabidopsis* cell wall proteoglycan consists of pectin and arabinoxylan covalently linked to an arabinogalactan protein. Plant Cell 25:270–287. https://doi.org/10.1105/tpc.112.107334

Tanimoto E, Fujii S, Yamamoto R, Inanaga S (2000) Measurement of viscoelastic properties of root cell walls affected by low pH in lateral roots of *Pisum sativum* L. Plant Soil 226:21–28. https://doi.org/10.1023/A:1026460308158

Tenhaken R (2015) Cell wall remodeling under abiotic stress. Front Plant Sci 5:771. https://doi.org/10.3389/fpls.2014.00771

Tyerman SD, Munns R, Fricke W, et al (2019) Energy costs of salinity tolerance in crop plants. New Phytol 221:25–29. https://doi.org/10.1111/nph.15555

van Zelm E, Zhang Y, Testerink C (2020) Salt tolerance mechanisms of plants. Annu Rev Plant Biol 71:403–433. https://doi.org/10.1146/annurev-arplant-050718-100005

Veselov DS, Sharipova GV, Akhiyarova GR, Kudoyarova GR (2009) Fast growth responses of barley and durum wheat plants to NaCl- and PEG-treatment: Resolving the relative contributions of water deficiency and ion toxicity. Plant Growth Regul 58:125–129. https://doi.org/10.1007/s10725-009-9359-y

Wang B, Sun Z, Yu Z (2020) Pectin degradation is an important determinant for alfalfa silage fermentation through the rescheduling of the bacterial community. Microorganisms 8:488. https://doi.org/10.3390/microorganisms8040488

Wang T, Park YB, Cosgrove DJ, Hong M (2015) Cellulose-pectin spatial contacts are inherent to never-dried *Arabidopsis* primary cell walls: evidence from solid-state nuclear magnetic resonance. Plant Physiol 168:871–884. https://doi.org/10.1104/pp.15.00665

Willats WGT, Mccartney L, Mackie W, Knox JP (2001) Pectin: Cell biology and prospects for functional analysis. Plant Mol Biol 47:9–27. https://doi.org/10.1023/A:1010662911148

Wolf S, Greiner S (2012) Growth control by cell wall pectins. Protoplasma 249:169–175. https://doi.org/10.1007/s00709-011-0371-5

Wu Y, Cosgrove DJ (2000) Adaptation of roots to low water potentials by changes in cell wall extensibility and cell wall proteins. J Exp Bot 51:1543–1553. https://doi.org/10.1093/jexbot/51.350.1543

Wu Y, Jeong BR, Fry SC, Boyer JS (2005) Change in XET activities, cell wall extensibility and hypocotyl elongation of soybean seedlings at low water potential. Planta 220:593–601. https://doi.org/10.1007/s00425-004-1369-4

Wu Y, Sharp RE, Durachko DM, Cosgrove DJ (1996) Growth maintenance of the maize primary root at low water potentials involves increases in cell-wall extension properties, expansin activity, and wall susceptibility to expansins. Plant Physiol 111:765–772. https://doi.org/10.1104/pp.111.3.765

Xiao C, Zhang T, Zheng Y, et al (2016) Xyloglucan deficiency disrupts microtubule stability and cellulose biosynthesis in *Arabidopsis*, altering cell growth and morphogenesis. Plant Physiol 170:234–249. https://doi.org/10.1104/pp.15.01395

Yan J, He H, Fang L, Zhang A (2018) Pectin methylesterase31 positively regulates salt stress tolerance in *Arabidopsis*. Biochem Biophys Res Commun 496:497–501. https://doi.org/10.1016/j.bbrc.2018.01.025

Yang JL, Zhu XF, Peng YX, et al (2011) Cell wall hemicellulose contributes significantly to aluminum adsorption and root growth in *Arabidopsis*. Plant Physiol 155:1885–1892. https://doi.org/10.1104/pp.111.172221

Zhang CB, Chen LH, Jiang J (2014) Why fine tree roots are stronger than thicker roots: The role of cellulose and lignin in relation to slope stability. Geomorphology 206:196–202. https://doi.org/10.1016/j.geomorph.2013.09.024

Zhang JF, Xu YQ, Dong JM, et al (2018) Genome-wide identification of wheat (*Triticum aestivum*) expansins and expansin expression analysis in cold-tolerant and cold-sensitive wheat cultivars. PLoS ONE 13:e0195138. https://doi.org/10.1371/journal.pone.0195138

Zhang SS, Sun L, Dong X, et al (2016a) Cellulose synthesis genes CESA6 and CSI1 are important for salt stress tolerance in Arabidopsis. J Integr Plant Biol 58:623–626. https://doi.org/10.1111/jipb.12442

Zhang T, Tang H, Vavylonis D, Cosgrove DJ (2019) Disentangling loosening from softening: insights into primary cell wall structure. Plant J 100:1101–1117. https://doi.org/10.1111/tpj.14519

Zhang Y, Liu Z, Khan AA, et al (2016b) Expression partitioning of homeologs and tandem duplications contribute to salt tolerance in wheat (Triticum aestivum L.). Sci Rep 6:1–10. https://doi.org/10.1038/srep21476

Zhong H, Lauchli A (1993) Changes of cell wall composition and polymer size in primary roots of cotton seedlings under high salinity. J Exp Bot 44:773–778. https://doi.org/10.1093/jxb/44.4.773

Zhu JK (2001) Plant salt tolerance. Trends Plant Sci 6:66–71. https://doi.org/10.1016/S1360-1385(00)01838-0

Zhu M, Shabala L, Cuin TA, et al (2016) Nax loci affect SOS1-like Na^+/H^+ exchanger expression and activity in wheat. J Exp Bot 67:835–844. https://doi.org/10.1093/jxb/erv493

Zhu T, Liang C, Meng Z, et al (2017) PrimerServer: a high-throughput primer design and specificity-checking platform. bioRxiv 181941. https://doi.org/10.1101/181941

Zhu XF, Wang ZW, Dong F, et al (2013) Exogenous auxin alleviates cadmium toxicity in *Arabidopsis thaliana* by stimulating synthesis of hemicellulose 1 and increasing the cadmium fixation capacity of root cell walls. J Hazard Mater 263:398–403. https://doi.org/10.1016/j.jhazmat.2013.09.018

Zidan I, Azaizeh H, Neumann PM (1990) Does salinity reduce growth in maize root epidermal cells by inhibiting their capacity for cell wall acidification? Plant Physiol 93:7–11. https://doi.org/10.1104/pp.93.1.7

Zörb C, Mühling KH, Kutschera U, Geilfus CM (2015) Salinity stiffens the epidermal cell walls of salt-stressed maize leaves: Is the epidermis growth-restricting? PLoS ONE 10:e0118406. https://doi.org/10.1371/journal.pone.0118406

Summary

Wheat (*Triticum aestivum* L.) production has been severely affected by soil salinization. There is need to improve wheat production in salinized soils. The importance of studying salinity tolerance mechanisms in wheat cannot be overemphasized. So far, wheat salinity tolerance has been studied from the aspects of osmotic adjustment, membrane transportation, hormone regulation, signal transduction, etc. However, studies on root cell walls were very limited. This study was conducted to investigate the salinity tolerance mechanisms of wheat, focusing on the function of root cell wall. The objectives of this study were to elucidate the interactions of cell wall composition, extensibility, expansin expression, root extension, and root growth under salinity stress and characteristics of root cell wall that contribute to root growth under salinity.

Two salt tolerant (JS-7, Xinchun-31 (XC-31)) and two salt sensitive (YL-15, GS-6058) spring wheat cultivars were selected as experimental materials. These cultivars were cultivated at 0 (control), 40, 80 and 120 mM NaCl concentrations. When root length showed significant difference among the cultivars, chemical compositions (pectin, hemicellulose I and II, cellulose, and uronic acid in each composition), extensibility, expansin expression and apoplastic pH in apical root (0-10 mm) cell walls, as well as cation exchange capacity of the whole root were investigated. The main results are described as follows:

1. Chemical compositions and properties of root cell wall in relation with root growth under salinity stress

Cultivars of JS-7 and XC-31 had higher root growth under salinity stress compared with YL-15 and GS-6058. This confirmed that the former is more tolerant to salinity stress than the latter. Salinity stress significantly decreased the pectin content in the elongation zone in all cultivars except JS-7. Hemicellulose I and II were significantly increased in the elongation and adjacent

zones in sensitive cultivars under salinity stress. Similarly, the cellulose content increased significantly across the cultivars in both root zones. This increment was more pronounced in the sensitive cultivars than in the tolerant cultivars. The uronic acid content in pectin in the elongation zone was decreased significantly in the sensitive cultivars relative to the tolerant cultivars, conversely, the uronic acid content in hemicellulose showed a reversed tendency. The cation exchange capacity of the root cell wall was significantly lower in sensitive cultivars than the tolerant cultivars. A positive correlation existed between root growth and relative content of pectin in elongation zone, and cation exchange capacity of the whole roots. However, root growth and relative content of cellulose were negatively correlated. These results indicate that a high pectin content and cation exchange capacity, as well as low hemicellulose and cellulose contents in the cell wall benefit root growth and thus, tolerance under salinity stress conditions.

2. Extensibility of root cell wall in relation with root growth under salinity stress

The extensibility of root cell wall was significantly decreased in sensitive cultivars, whereas, that in tolerant cultivars was maintained at the same level as that in the control. Root extension and the differences between cultivars were largely dependent on elastic extension, which accounted for one-half to two-thirds of the total extension. Viscosity and the plastic extension of the root cell walls had no difference across the treatments and cultivars. The significant decrease in cell wall elasticity in the root elongation region was one of the factors that depressed root growth in sensitive cultivars under salt stress. The well-maintained elasticity of tolerant cultivars alleviated the depression of root growth by NaCl. Cell wall elasticity was positively correlated with the relative pectin and hemicellulose I contents and negatively correlated with the relative cellulose content. Under saline conditions, the relative hemicellulose II content was not altered in the sensitive cultivars; however, it decreased

significantly in the tolerant cultivars. Therefore, changes in chemical composition of cell wall corresponded with the cell wall extensibility and root growth in wheat cultivars at different levels of salinity tolerance.

Salinity decreased the root cell wall extension significantly, especially in sensitive cultivars through an increased extension resistance, but, there were no significant effects on the tolerant cultivars. The elastic properties of root cell wall of wheat under salinity were more pronounced in root elongation as compared with the plastic properties. The increment in pectin and hemicellulose I better improved the elastic extension in the root cell wall, relative to the deposition of cellulose.

3. Specific expression of expansins in response to apoplastic pH under salinity stress

Salinity treatment significantly reduced apoplastic pH in apical root in both tolerant and sensitive cultivars. The apoplastic pH in elongation zone was about 6.28 under non-saline condition, while it decreased to about 5.3 under salinity in both cultivars. For the roots grown under the non-saline condition, the optimal pH for cell wall extension was 6.0 and 4.6 in tolerant and sensitive cultivars, respectively. In contrast, roots grown under salinity showed that the optimal pH for cell wall extension was 5.0 in the tolerant and 6.0 in the sensitive cultivars. Therefore, the apoplastic pH (5.3) under salinity was favorable to root extension in tolerant cultivars, but not in the sensitive ones. Expansin gene expressions in root cell wall were generally suppressed by salinity. Gene expressions of *TaEXPA3*, *TaEXPA6, TaEXPB1* and *TaEXPB10* were reduced in both cultivars under salinity stress. However, those of *TaEXPA5* and *TaEXPA8* in tolerant cultivars were increased under salinity. This increment may improve root extension under salinity. The expansin activity of the tolerant cultivar was significantly higher than that of the sensitive one. *TaEXPA8* mediated cell wall loosening especially at pH 5.0, whereas, *TaEXPA5* activated especially at pH 6.0. Under salinity stress

with lowered apoplastic pH, expansins in the tolerant cultivar resulted in the maintenance of cell wall extensibility, whereas those in the sensitive cultivars had no such activity.

This study investigated the root cell wall from the aspects of chemical composition, physical property and expansin expression. Each component of the root cell wall has its own effect on root extension. The extension of root cell wall under saline conditions, with reduced turgor pressure, was adversely depressed in the sensitive cultivars, but maintained to some extent in the tolerant ones. The wall loosening corresponded to the elastic extension, which involved wall expansins and all other components. When the cell wall loosens, new wall materials fill in the space or bind to the old wall. These materials correspond with the plastic nature, i.e. the final elongation of the root.

The present study revealed the regulation role of cell wall in root growth. Cultivar differences in salinity tolerance can be related to the property of root cell wall. Characteristics of root cell wall such as higher amount of uronic acids and pectin, lower amount of cellulose, and specific expansin expression were of importance for root extension and growth under saline stress.

論文要旨

コムギ (*Triticum aestivum* L.) の生産は、土壌の塩類化によって深刻な被害を受ける。そこで塩害土壌でのコムギ生産を改善する必要がある。コムギの耐塩性メカニズムを研究することが極めて重要である。これまで、コムギの耐塩性は、浸透圧調節、膜輸送、ホルモン調節、シグナル伝達などの側面から研究されてきたが、根の細胞壁に関する研究は非常に限られていた。本研究では、コムギの耐塩性メカニズムを根の細胞壁の機能に着目した。本研究の目的は、塩ストレス下での細胞壁組成、伸展性、エクスパンシンの発現、根伸長、根の成長との相互作用および塩ストレス下での根の成長に寄与する根細胞壁の特性を明らかにすることである。

春コムギの耐塩性 2 品種（JS-7 と Xinchun-31（XC-31））と塩感受性 2 品種（YL-15 と GS-6058）を供試した。これらの品種は、0（対照区）、40、80、および 120 mM の NaCl 濃度条件で栽培した。根長に品種間で有意差があった時、化学組成（ペクチン、ヘミセルロース I および II、セルロースおよび各成分におけるウロン酸）、伸展性、エクスパンシンの発現、および根端（0-10 mm）細胞壁の pH、根全体の陽イオン交換容量を調査した。以下に主な結果を示す。

1. 塩ストレス下での根細胞壁の化学組成・性質と根の成長の関係

JS-7 と XC-31 の品種は、YL-15 と GS-6058 と比較して、塩ストレス下でより高い根の成長を示し、前者は後者よりも塩ストレスに耐性があることが確認された。塩ストレスは、JS-7 を除くすべての品種で伸長域のペクチン含有量が大幅に減少した。ヘミセルロース I および II は、塩ストレス下における塩感受性品種の根伸長域と隣接域で有意に増加した。同様に、セルロース含有量は、すべての品種で根端におい

て増加し、この増加は、耐塩性品種よりも塩感受性品種でより顕著であった。根伸長域のペクチン中のウロン酸含有量は、耐性品種に比べて感受性品種で有意に減少し、ヘミセルロース中のウロン酸含有量は逆の傾向を示した。根細胞壁の陽イオン交換容量は、耐塩性品種よりも塩感受性品種で有意に低かった。根の成長と伸長域におけるペクチンの相対的含有量と根全体の陽イオン交換容量との間には正の相関があった。ただし、根の成長とセルロースの相対的含有量との間には負の相関関係があった。これらの結果は、根の成長には、ペクチン含有量と陽イオン交換容量が高いこと、細胞壁のヘミセルロースとセルロース含有量が低いことが有益であり、塩ストレス条件下での耐性に寄与することが示唆された。

2. 塩ストレス下での根細胞壁の伸展性と根の成長の関係

根細胞壁の伸展性は塩感受性品種で有意に低下したが、耐塩性品種では対照区と同程度に維持された。根の伸長と品種間差異は、全体の伸長の 1/2 から 2/3 を占める弾性伸長に大きく依存した。根の細胞壁の粘性と塑性伸長は、処理と品種間で違いはなかった。根伸長域における細胞壁弾性の有意な減少は、塩ストレス下での感受性品種の根の成長を抑制した一要因であった。耐性品種の細胞壁弾性の維持は、塩ストレスによる根の成長抑制を緩和した。細胞壁の弾性は、相対的なペクチンおよびヘミセルロース I 含有量と正の相関関係があり、相対的なセルロース含有量と負の相関関係があった。塩ストレス条件下では、感受性品種のヘミセルロース II 含有量の相対値は変化しなかったが、耐性品種では大幅に減少した。したがって、細胞壁の化学組成の変化は、耐塩性が異なるコムギ品種の細胞壁伸展性および根の成長に対応することが示された。

塩分は，根の細胞壁の伸長を有意に減少させ，特に感受性の高い品種では伸長抵抗が増加したが，耐性の高い品種には有意な影響はなかった。塩条件下におけるコムギの根部細胞壁の弾性は，塑性と比較して，根の伸長にとってはより顕著な影響があった。セルロースの付着に対して，ペクチンとヘミセルロースIの増加は，根の細胞壁の弾性伸長を改善した。

3. 塩ストレス下でのアポプラスト pH に応答したエクスパンシンの特異的発現

塩処理は、耐塩性品種と塩感受性品種の両方で、根端部のアポプラスト pH を大幅に低下させた。伸長域のアポプラスト pH は、非塩ストレス条件下では約 6.28 であったが、塩ストレス条件下では両品種で約5.3に低下した。非塩ストレス条件下で成長した根の場合、細胞壁伸長に最適なpHは、耐性品種で6.0、感受性品種で4.6であった。対照的に、塩ストレス条件下で成長した根は、細胞壁伸長に最適なpHは、耐性品種で 5.0、感受性品種で 6.0 であった。したがって、塩ストレス条件下でのアポプラスト pH（5.3）は、塩感受性品種より耐塩性品種における根の伸長に有利であった。細胞壁の組織の緩みを媒介するエクスパンシン遺伝子の発現は、塩ストレスによって抑制された。*TaEXPA3*、*TaEXPA6*、*TaEXPB1*、*TaEXPB10* の遺伝子の発現は、塩ストレスにより両品種で抑制された。しかし、耐塩性品種の *TaEXPA5* と *TaEXPA8* の遺伝子の発現は、塩ストレスにより増加した。この発現上昇によって、塩ストレス条件下での根の伸長が改善されたと推測される。耐性品種のエクスパンシン活性は感受性品種より有意に高かった。*TaEXPA8* は pH 5.0 で、*TaEXPA5* は pH 6.0 で発現が活性化した。アポプラスト pH が低下する塩ストレス下では、耐性品種のエクスパンシンは細胞壁の伸展性を維持できたが、感受性品種のエクスパンシンはこのような効果は見られなかった。

本研究では、根の細胞壁について、化学組成、物性、エクスパンシン発現の観点から調査した。根の細胞壁の各構成成分は、根の伸長にそれぞれ影響を及ぼす。塩ストレス条件下での膨圧低下による根細胞壁の伸長は、感受性品種では抑制されたが、耐性品種ではある程度維持された。細胞壁の緩みは、細胞壁のエクスパンシンをはじめとするすべての構成要素が関与する弾性伸長と相応した。細胞壁が緩むと、新しい細胞壁の材料がスペースを埋めるか、古い細胞壁に結合する。これらの材料は、塑性的な性質、つまり根の最終的な伸長に相応する。

本研究では、塩ステレス下でのコムギの根の成長における細胞壁の生理生化学的特性の調節機能を明らかにした。コムギの耐塩性は、根の細胞壁の特性に関係しており、塩ストレスかでの根の成長には、ウロン酸やペクチン含有量の増加、セルロース含有量の減少，特異的なエクパンシンの発現が重要であることが明かとなった。

Publication list

Paper 1: Related to chapter 2.

Title	Effects of sodium chloride on root growth, cell wall composition, and cation exchange capacity of wheat cultivars varying in salt tolerance
Authorship	Yang Shao, Irshad Muhammad, Cuihua Huang, Xian Xue, Yunus Qiman, and Ping An
Full Name of Scientific Journal	Sand Dune Research 68 (1)
Published date or accepted date	September, 2020 (June, 2021)

Paper 2: Related to chapter 3.

Title	Differential responses of roots for varying tolerance to salinity stress in wheat with special reference to elasticity
Authorship	Yang Shao, Ping An, Xiaohui Feng, Irshad Muhammad, Victoria Otie, Weiqiang Li, Yuanrun Zheng, and Yunus Qiman
Full Name of Scientific Journal	Plant Growth Regulation 94 (2): 183-193
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