Salinity stress-induced modification of pectin activates stress signaling pathways and requires HERK/THE and FER to attenuate the response

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Abstract

Soil salinity is an increasing worldwide problem for agriculture, affecting plant growth and yield. In our attempt to understand the molecular mechanisms activated in response to salt in plants, we investigated the *Catharanthus roseus* Receptor like Kinase 1 Like (CrRLK1L) family, which contains well described sensors previously shown to be involved in maintaining and sensing the structural integrity of the cell walls. We have observed that *herk1the1-4* double mutants, lacking the function of the *Arabidopsis thaliana* Receptor like Kinase HERKULES1 combined with a gain of function allele of THESEUS1, phenocopied the phenotypes previously reported in plants lacking FERONIA (FER) function. We report that both *fer-4* and *herk1the1-4* mutants respond strongly to salt application, resulting in a more intense activation of early and late stress responses. We also show that salt triggers de-methyl esterification of loosely bound pectins. These cell wall modifications might be partly responsible for the activation of the signaling pathways required to activate salt stress responses. In fact, by adding calcium chloride or by chemically inhibiting pectin methyl esterase (PME) activity we observed reduced activation of the early signaling protein Mitogen Activated Protein Kinase 6 (MPK6) as well as a reduced amplitude in salt-induced marker gene induction. We show that MPK6 is required for the full induction of the salt-induced gene expression markers we tested. However, the sodium specific root halotropism response is likely regulated by a different branch of the pathway being independent of MPK6 or calcium application but influenced by the cell wall sensors FER/HERK1/THE1-4 and PME activity. We hypothesize a model where salt-triggered modification of pectin requires the functionality of FER alone or the HERK1/THE1 combination to attenuate salt responses. Collectively, our results show the complexity of salt stress responses and salt sensing mechanisms and their connection to cell wall modifications, likely being in part responsible for the response phenotypes observed in salt treated plants.

Introduction

In plants, cell walls act as a barrier that protects plants to environmental threats and functions as structural support for the whole plant body [1]. In young Arabidopsis plants, primary cell walls (CWs) are made of complex polysaccharide networks where the main load bearing component is cellulose, a linear polymer composed of amorphous and crystalized ß 1-4 linked glucose monomers. Cellulose fibrils are intimately connected with pectin, another major component of the cell wall, described to be essential for regulating cell expansion and elasticity (reviewed in [2]). Contrary to cellulose, which is synthetized at the plasma membrane level, pectins are synthetized in Golgi bodies and released in the apoplast in a highly methylated form [3], [4]. Cell walls are subjected to controlled modifications that allow the cell to extend, driving the direction of cell growth (reviewed in [5]). Cell wall modifications also happen in response to both biotic and abiotic stresses and these structural changes are perceived and sensed by plants. Receptors/sensors are responsible for triggering intracellular downstream signals in response to turgor pressure driven cell wall-dependent mechanical distortions, a process called cell wall integrity (CWI) maintenance [6], [7].

Several members of the *Catharanthus roseus* Receptor Like Kinase1 Like (CrRLK1L) family have been described to play a role in mechanisms related to cell wall remodeling (*e.g.* cellulose impairment, pathogen infections, fertility) [8]. CrRLK1Ls contain 2 malectin domains, proposed to selectively bind complex carbohydrates, and an intracellular kinase domain responsible for transmitting signals intracellularly [9]. In Arabidopsis, 17 genes encoding for CrRLK1Ls have been identified and in some cases their functional roles have extensively been studied. For example FERONIA (FER), appears to be vital in multiple aspects of plant life (*e.g.* fertility, root hair development, mechanical stress, immune responses) while THESEUS1 (THE1), HERKULES 1/2 (HERK1/2) and CURVY (CVY1) are essential for cell elongation and cell morphology related processes [8], [10], [11].

Even if CrRLK1Ls are extensively studied, the mechanism through which they work is not fully understood. It seems that CrRLK1Ls might form complexes through transient hetero-oligomerization as found for the newly identified LETUM1/LETUM2 modulating plant autoimmunity and HERK1/ANJEA or ANXUR (ANX)/BUDDHA'S PAPER SEAL (BUPS) that control pollen tube related mechanisms [12], [13]. CrRLK1Ls also act as receptors for small extracellular peptides called Rapid Alkalinization Factors (RALFs). In Arabidopsis, 34 different *RALF* genes have been identified and RALF22, 23, 33, 34 and RALF1 have been reported to function as FER ligands, with the latter being required for Arabidopsis ovule fertilization [12], [14]–[16]. On the other hand, THESEUS1 (THE1) seems to be the receptor of RALF34, and both are essential for regulating lateral root development in Arabidopsis [16]. Some CrRLK1Ls have been defined being responsible for sensing cell wall alterations and mechanical stress responses with THESEUS1 (THE1) being essential to fully activate at least one branch of the CWI signaling pathways [17], [18]. In Arabidopsis several other proteins have been characterized to play a role in CWI signaling, including stretch-activated plasma membrane-localized calcium channels (MCAs) and the Nitrate Reductase 1 and 2 (NIA1/2), the latter being recently described to be play an essential role in the cell wall damage dependent cell cycle regulation [2], [5], [17], [18].

Interestingly, many of the CWI players have been found to overlap with salt stress response regulators. This is the case for Leucin Rich Repeat Receptor Like Kinases MIK2/KISS (MALE DISCOVERER 1-INTERACTING), FEI1/2, FERONIA, but also for NIAs and MCAs [19], [20, p. 5], [21]–[23]. Intriguingly, while loss of function mutants for *mik2*, *fei1/2*, *nia1/2* and *mcas* (*mca1,2*) were reported to be highly sensitive to salt application, their responses to cellulose induced cell wall damage varied, with *fei1/2* being extremely sensitive and *mik2*, *nia1/2* and *mca1*, being partly or almost completely insensitive to cellulose inhibition [17], [18], [20]–[23]. Yet how and why CWI and salt induced signaling overlap has been poorly investigated.

For decades, researchers have tried to discover how plants sense salt. Salt application simultaneously induces ionic and osmotic stress, which both trigger subsequential general and specific signaling responses. Within seconds to

minutes after salt treatment, early signaling molecules are induced, such as calcium spikes, reactive oxygen species (ROS) accumulation and mitogen protein kinase (MAPK) cascade phosphorylation (reviewed in [24], [25]). Only recently, the identification of MONOCATION-INDUCED $[Ca^{2+}]_i$ INCREASES 1 (MOCA1) has proposed that salt is sensed at the plasma membrane. MOCA produces glycosyl inositol phosphorylceramide (GIPC) sphingolipids, able to bind to monovalent cations (such as Na^{2+}), triggering the opening of unknown plasma membrane localized Ca^{2+} channels involved in calcium spikes burst [26]. The accumulation of intracellular calcium is necessary to activate the salt-overly-sensitive (SOS) pathway, which consists of the Na^+/H^+ exchanger SOS1 and SOS2/3 signaling components, all required for limiting the damaging effects of salt on cells by increasing Na^+ efflux [27]–[29]. On the other hand, the change in main root direction observed in high salt concentrations, called halotropism, was actually stronger in the *sos* mutants, and was shown to be dependent on auxin distribution [30], [31]. Interestingly, salt-stress induced calcium spikes depend on FER, as *fer-4* loss of function mutants are unable to induce calcium bursts as wild type plants [19]. FERONIA seems to be required to maintain cell wall architecture in response to salt stress since loss of function *fer-4* mutants display cell bursting and severe cell swelling in the root in the presence of high salt concentrations. It has been proposed that the salt oversensitive phenotypes observed in FER mutants depend on the enhanced cell wall softening due to the presence of cell wall localized sodium ions. These results are corroborated by supporting data showing that FER's malectin domain can bind pectin macromolecules *in vitro* [19]. Exogenous calcium and boron application, can complement, to some extent, FER-dependent salt-induced phenotypes suggesting that salt application might affect pectin architecture [19]. No available information exists for a role of other CrRLK1L members in sensing salt induced cell wall damage. However, other cell wall localized proteins such as the Leucine-rich repeat extensins (LRX) 3/4/5 have been reported be involved in helping the physical association between RALF22/23 and FERONIA causing FER inactivation through its internalization upon salt stress [32].

Here we report that salt changes cell wall architecture by de-methyl esterification of pectin, which in turn is proposed to be responsible for a range of early cellular signaling, gene expression and phenotypic responses. In our attempt to expand our current knowledge on the role of other CrRLK1L in the abiotic field, we have analyzed a complete set of salt stress responses to shed some lights on the insight of early and late salt stress signaling events in previously characterized mutants. We also show that while loss of function mutants lacking THE1 or HERK1 alone show wild type like salt stress responses, plants lacking both HERK1 in combination with a truncated, gain of function allele version of THESEUS1 (*herk1the1-4* mutants [33]) show severe salt sensitivity, similarly to that of *fer-4* loss of function mutants. FER and HERK1/THE1-4 are required for dampening the salt-induced Mitogen Activated protein Kinase 6 (MPK6) activation, as higher activation of MPK6 is observed in *fer*-4 and *herk1the1-4* mutants which is consistent with the higher expression of salt induced, cell wall damage dependent, marker genes in these mutants. We also report that the salt-induced pectin methyl esterification of loosely bound pectins can be rescued by the pectin methyl esterase inhibitor [(-)-Epigallocatechin gallate (EGCG)] or calcium chloride ($CaCl_2$) application. Downstream of the cell wall changes, we describe a branched pathway where cell wall changes are sensed by FER, HERK1/THE1-4 combination, to inhibit both MPK6- dependent gene expression and MPK6 and calcium-independent halotropic responses. Collectively, we show that salt changes the degree of pectin methyl esterification, likely affecting the structural integrity and the mechanical properties of the cell wall, sensed by known sensors which are in turn responsible for regulating a range of salt stress induced responses.

Results

herk1the1-4 and fer-4 mutants are affected in early responses to salt

To investigate whether other CrRLK1Ls, besides *fer-4,* are disturbed by salt application [19], [32], a collection of available mutants in members previously implicated in CWI sensing (Table S1), were directly germinated and grown in the presence/absence of 150 mM NaCl for 10 days (Figure S1A). No bleaching was observed in control plates (not shown) and the differences between the genotypes were only quantified in salt containing plates (Figure 1A). After 10 days of growth, *fer-4* mutants showed higher rates of cotyledon bleaching as expected, while both *herk1* and *the1-1* single knock-out (KO) mutants displayed comparable salt induced cotyledon bleaching to that observed in salt-grown wild type (wt) controls. On the other hand, slightly more bleaching was discovered in the single *the1-4* mutant that expresses a truncated THE1 protein, and was previously described as a gain of function allele of THE1 [34]. Interestingly, the double *herk1the1-4* showed enhanced bleaching comparable to *fer-4* (Figure 1A), suggesting that a combination of HERK1 and THE1-4 is required to regulate salt-dependent sensitivity. Genetically combining *the1-1* loss of function and *fer-4* mutant led to the same bleaching phenotype as *fer-4* single mutants (Figure 1A/S1A) suggesting that the FER-dependent salt induced phenotype cannot be alleviated by impairing the functionality of the cell wall damage sensor THE1. To better investigate the role of CrRLK1Ls mutants in salt induced signaling, different experimental set-ups have been employed to analyze early (MPK6 phosphorylation, gene expression) and late responses (root halotropism, cotyledon bleaching and cell wall composition) to salt stress (Figure 1B).

To understand whether salt-triggered oversensitive phenotypes observed in *fer-4* as well as in *herk1the1-4* are the result of enhanced salt-dependent signaling we analyzed early responses upon salt application. In salt treated plants, one of the early responses is the phosphorylation of MPK6 [35]. In plants and animals, MAPK cascades are initiated by a specific stimulus which triggers the activation of MAPK kinase kinases (MAPKKKs), phosphorylating a MAPK kinase (MAPKK) and in turn leading to the activation of MAPKs, also known, in Arabidopsis as MPKs [36]. By using a well characterized and widely used antibody [37]–[39] that recognizes the phosphorylated form of both Arabidopsis MPK6 and 3, we have analyzed salt induced MPK6 phosphorylation upon 15 minutes of treatment. We first performed control experiments in a previously characterized MPK6 allele (*mpk6-3*) [35] to confirm the specificity of the antibody (Figure S1B). Then we used the same set-up to perform analysis in the mutants. Both *fer-4* and *herk1the1-4* mutant lines showed a stronger MPK6 activation when compared to NaCl-treated wt seedlings (Figure 1C/ S1C). Interestingly, similar to the bleaching phenotype, while *herk1* and *the1-1* did not show a difference with salt-treated wt control, *the1-4* displayed enhanced salt-induced MPK6 activation when compared to the wt, although less evident when compared to the corresponding double *herk1the1-4* mutants or *fer-4* (Figure S1C).

To investigate whether the lack of FER or HERK1/THE1-4 had an effect on salt-induced gene expression levels, we selected three salt-induced marker genes [17], [40]–[42] and checked their expression in response to salt application. We chose *ProPEP3*, *WRKY40* and *RRTF1* because they are strongly induced within 1 h from salt application and because they have been described responsive to a wide number of salt dependent modifications, like cell wall changes, ABA-dependent signaling and reactive oxygen species (ROS) [17], [40]–[42] (Table S2). We treated wt, *fer-4* and *herk1the1-4* seedlings for 1 h and found that the expression level of salt responsive genes was higher in response to salt in the mutant lines when compared to the wt controls (Figure 1D). Similar to the cotyledon bleaching rates, *the1-4* but not *the1-1*, showed enhanced transcriptional regulation of salt marker genes compared to salt treated wt seedlings (Figure S1D).

Since *mpk6-3* loss of function mutants were reported to show reduced root growth inhibition in response to salt stress [43], likely associated to reduced salt sensitivity, we analyzed the expression pattern of the selected gene markers in the *mpk6-3* mutant background and observed that MPK6 is at least in part responsible for the upregulation of *PROPEP3*, *RRTF1* and *WRKY40* by salt (Figure 1E). The results suggest that the enhanced MPK6 activation observed in

fer-4 and *herk1the1-4* might also be responsible for their amplified susceptibility to salt. Recently it was reported that ectopic over expression of *RRTF1* is detrimental to plant salt tolerance [42]. This is consistent with our observations, where the higher *RRTF1* expression pattern described in both salt-treated *fer-4* or *herk1the1-4* is linked to the higher plant salt susceptibility (Figure 1D/S1D).

herk1the1-4 and fer-4 mutants are affected in late-MPK6 independent responses to salt

To evaluate if FER and HERK1/THE1-4 regulate also other salt responses we analyzed later responses such as root growth and halotropism. When facing a salt gradient, plant roots bend to avoid high salt concentrations, a phenotype referred to as halotropism [30], [31], [44]. Wild type, *fer-4* and *herk1the1-4* double mutant seedlings were subjected to diagonal (mock or salt) gradients and root bending [(angle) º] was measured 24 h after salt application. As shown in Figure 2A (biological replicates and root elongation rates are shown in Figure S2A/B/C) when compared to the wt *fer-4* exhibited enhanced halotropism-triggered root bending, while *herk1the1-4* showed an intermediate phenotype (and was found statistically different from the wt in Figure S3B/4F). Interestingly, salt dependent root halotropism responses do not require MPK6 (Figure 2B) suggesting the presence of at least 2 branches downstream of FER/THE/HERK, one of which is MPK6-independent.

Next, to have a better understanding of the growth dynamics of *fer-4* and the double mutant *herk1the1-4*, we grew seedlings in vertical plates for 4 days and transferred them onto plates containing homogeneous NaCl (or no NaCl (control). To have a complete overview of both salt-triggered early and late effects, salt induced root growth inhibition was traced over 42 h in a specialized growth chamber where time lapse photography-based analyses were performed over time (Figure 2C). Previously, it has been reported that wt seedlings subjected to salt stress display a "multi-phasic" trend of growth, which is slowly inhibited within 8 h from initial salt application (quiescent phase) followed by a recovery phase [45]. While the mutants still showed a quiescence response similar to wt, both *fer-4* and *herk1the1-4* mutants show drastic root growth and elongation rate reduction starting from 30 h (*fer-4*) and 32 h (*herk1the1-4*) upon salt application (Figure 2C). To evaluate whether the higher salt sensitivity observed in the double *herk1the1-4* was also present in adult plants we investigated the responses to salt in soil-grown plants (Figure S2D). For this assay, Col-0, *fer-4* and *herk1the1-4* plants were once watered with MilliQ water or salt (75 mM) and then rosette fresh weight was measured at the end of the treatment. Fresh weight quantification showed a significant difference between salt treated wt and the two mutant lines (Figure S2E) while reduced biomass was also observed in the control conditions (Figure S2F). Together, these results show that the *herk1the1-4* mutant exhibits similar enhanced salt sensitivity as the earlier described *fer* mutant in immediate, early and late salt responses.

Salt-induced early but not late responses are mitigated by calcium application in Arabidopsis seedlings

The effect of calcium in rescuing *fer-4* dependent salt induced root growth phenotypes was suggested to be associated to calcium mediated pectin crosslink increase, being FER's malectin domain reported to bind pectins *in vitro* [19]. To assess the effect of Ca^{2+} on signature responses to salt, such as early and late cellular and physiological responses, we treated seedlings with salt alone or in combination with 5 mM $CaCl_2$. First, to understand whether calcium can influence the growth dynamics and intensity of the salt-triggered quiescent phase, we performed a time course analysis on wt seedlings grown in vertical conditions and transferred after 4 days on salt-containing plates (Figure 3A). We observed that, as expected, salt induces an early drop in root elongation for the first hours upon salt application followed by a recovery phase that is fully completed at 10 h from the application of the stimulus. When calcium and salt were added together in the medium, we detected a shorter and less severe quiescent phase and an early recovery phase (Figure 3A).

When analyzing early salt induced signaling response, we found that already after 15 minutes calcium could alleviate NaCl triggered MPK6 phosphorylation in the wt as well as in the salt oversensitive mutants *fer-4*, *herk1the1-4* (Figure 3B and Figure S3A). Also, when wt, *fer-4* and *herk1the1-4* mutant lines were treated with a NaCl/$CaCl_2$ combination, gene expression level was rescued to the same (*PROPEP3*, *WRKY40*) or almost similar (*RRTF1*) physiological expression levels when compared to the mock controls (NaCl/$CaCl_2$ vs mock) (Figure 3C). As previously reported, sodium dependent root halotropism is not mitigated by exogenous calcium application (Figure S3B/C, and [31]). Thus, while Ca^{2+} has been suggested before to function as a cell wall strengthening agent which can mitigate salt induced *fer-4* cell swelling phenotypes, we now show that its effects are likely to happen earlier in time not only on a structural, but also on a cellular signaling level.

Salt induced homogalacturonan de-methyl esterification is partly required to activate salt-triggered responses

In cell walls, calcium ions have been connected to enhanced wall strength, and described to play a role in the formation of calcium ion-mediated homogalacturonan-homogalacturonan (HG, the main pectin component) crosslinks [46]. For this reason, the hypotheses for calcium being responsible to alleviate salt induced responses have been associated to its ability to alter pectin architecture. To study salt induced cell wall modifications directly, we analyzed pectin isolated from 6-day old wt seedlings treated with or without salt for 24 h. The extracted cell wall material was subjected to pectin extraction by using boiling distilled water (see Material and Methods). Total sugars in these extracts were quantified [47], [48] and sugars were spotted on nitrocellulose membrane to perform dot blot analyses. To examine in detail the structural organization of pectins and in particular the degree of methylation (DM) of HG we used three cell wall probes, namely 2F4, LM19 and LM20 which respectively recognize partially de-methyl-esterified, fully de-methyl-esterified and highly methyl-esterified HG [49], [50]. As a control, HG methyl esters were removed by incubating the same amount of total sugars spotted in the upper part of the dot blot panels (native pectins) with high pH buffer (Na_2CO_3, pH 11, (deM-HG)) providing loading controls for both 2F4 and LM19 and negative controls for LM20 specificity. 2F4, a probe previously reported to specifically recognize Ca^{2+}-crosslinked HG dimers [51], [52] shows enhanced signals in the Na_2CO_3 treated extracted sugars (Figure 4A, first lower panel), suggesting that this probe in fact binds partly or fully de-methyl esterified HG [as also reported in [49]]. When wt seedlings were treated with salt, we observed that water-extracted cell wall pectins displayed enhanced 2F4 and slightly enhanced LM19 signal, while no differences were detected in LM20 incubated samples, revealing an effect for salt in altering pectin methyl esterification patterns by decreasing the concentration of methyl esters on the homogalacturonan (Figure 4A). Analysis of pectin methyl esterification in the salt sensitive *fer-4* and *herk1the1-4* mutants showed that similarly to the wt they also display changes in the overall methyl esterification distribution upon salt stress suggesting that in these mutants structural modifications of the cell walls were also induced by salt treatments similar to wt but were not detectable in mock conditions (Figure 4B/S4A).

To investigate the cellular and physiological consequences of salt induced HG de-methyl esterification, a chemical PME inhibitor, (-)-Epigallocatechin gallate (EGCG), was used to perturbate PME activity [53]. Therefore, seedlings of wt, *fer-4* and *herk1the1-4* were treated with a combination of salt and ECGC and effects were compared to the mock (DMSO; EGCG solvent). We found that even if ECGC alone did not affect MPK6 activation in any of the genotypes, when the combination NaCl/EGCG was applied, MPK6 activation was reduced especially in the salt sensitive mutants (Figure 4C, S4B), suggesting that inhibition of salt-induced de-methyl-esterification of pectin can alleviate the immediate stress responses reported in this study. Similarly, to determine the effect of EGCG on salt-induced gene expression levels, seedlings of Col-0, *fer-4* and *herk1the1-4* mutants were treated with NaCl, EGCG or NaCl/EGCG. During these experiments we observed a similar trend as in seedlings treated with $CaCl_2$/NaCl combinations. Even if EGCG itself did not affect the expression of the selected markers, when EGCG was applied in combination with NaCl,

gene expression induction levels were reduced most evidently in the salt sensitive *fer-4* and *herk1the1-4* mutants (Figure 4D).

To study the effect of EGCG on root halotropic response, we analyzed root angle in the halotropism assay, performed on seedlings treated by NaCl on a gradient supplemented with EGCG. EGCG itself did not affect root direction at any time point when compared to the mock control (Figure 4E, black vs white bars) but during salt stress, the enhanced halotropic phenotypes of both *fer-4* and *herk1the1-4* were significantly reduced by EGCG supplementation. The effect of EGCG was not statistically significant in Col-0 at any of the analyzed stages while for *fer-4* and *herk1the1-4* its effect was evident after 48 h of treatment [(Figure 4E), biological replicates and root elongation rates are shown in Figure S4C/S4D/S4E] likely being dependent on the diffusion speed of the chemical vs the diffusion of NaCl in the gradient. To confirm that EGCG could alleviate the salt-induced pectin de-methyl esterification we analyzed HG methylation patterns and discovered that when both salt and EGCG were present the amount of de-methyl esterified pectin recognized by 2F4 (Figure 4F) was reduced compared to that detected in seedlings treated with salt only, while LM20 signal was not affected as expected (Figure S4F). Thus, ECGC treatment increases the amount of partially demethylated pectin but does not alter the amount of highly methylated pectin under salt stress. Interestingly, when a similar experiment was performed where wt seedlings were treated with a combination of salt and calcium (Figure 4G), calcium was also found to attenuate the 2F4 dependent signals, suggesting that salt-dependent de-methylation of HG might lead to reduced cell wall strength which can be reinforced by exogenous application of calcium, and/or that calcium would work indirectly to affect the regulation of enzymes required for the salt induced pectin relaxation. Yet, these hypotheses remain to be addressed.

Taken together these results suggest that salt-induced modification at the cell wall level involves pectin architecture alterations and that PME inhibition can partially counteract protein kinase phosphorylation and salt dependent gene expression in salt sensitive genotypes. Even if our cell wall analyses were performed 24 h after salt application, it is possible that changes in pectin moieties happen quickly at the single cell level. However, by using our method, they become only detectable later upon the treatment, when the effect on pectin is widely spread in all cell types. This hypothesis would explain why salt induced intracellular responses can be attenuated by Ca^{2+}/EGCG application. Moreover, while MPK6 and calcium could not mitigate salt induced root halotropism, likely controlling another branch of the salt induced stress responses, EGCG was able to soften the enhanced root bending phenotypes observed in salt treated salt oversensitive mutants, suggesting that pectin architecture modification might be responsible for a large part of the salt induced responses in plants.

Discussion

Cell walls are complex polysaccharide networks composed of cellulose, hemicelluloses, pectins and structural proteins [54]. Pectins are abundant polysaccharides in Arabidopsis primary cell walls, that are rich in galacturonic acid and create homogalacturonan (HG) chains made of linear α-1-4-linked galacturonic acid monomers. HG is transported to the cell wall in a highly methylated form and de-methyl esterified in the apoplast by the action of pectin methyl esterases (PMEs) [55]. It has been suggested that de-methyl esterified HG can be crosslinked by Ca^{2+} and form so-called egg-boxes, pectin crosslinked structures involved in promoting cell wall stiffening [56]. However, the hypothesis that calcium crosslinks limit cell growth is not well supported, as the analysis of calcium mediated pectin crosslinks is challenging [57]. In this study we report that external calcium application to salt treated seedlings rather enhanced root growth (Figure 2A). It has been reported that pectin modifications and crosslinking influence the strength of the wall as well as cell shapes [7], [58]. Yet, while in pollen tubes the data fit the hypothesis that lower HG esterification is associated with higher cell wall stiffening, several other studies show that reduction of HG methyl esterification patterns rather enhance cell wall expansion [59], [60]. Analysis performed by atomic force microscope (AFM)-based indentation on shoot apical

meristems of plants with reduced pectin methyl esterification, showed enhanced cell wall softening [59], [60]. It has been hypothesized that in the absence of calcium, de-methyl esterified pectins displayed altered fluidity, being more susceptible to the action of pectin degrading enzymes like polygalacturonanses (PGs) which enhance cell wall extensibility [49], [57].

In recent years a mechanism required to monitor cell wall structure named cell wall integrity (CWI) maintenance has been described in plants. CWI is essential for maintaining cell wall plasticity and initiating responses to cell wall damage (CWD), which can be triggered by both biotic or abiotic stress [5]. Cell wall damage sensor candidates include receptor-like kinases (RLKs), Leucin-rich repeat extensins (LRXs) and ion channels [reviewed in [7]]. Well-described CWI sensors belong to the *Catharanthus roseus* Receptor-Like Kinase1-Like (CrRLK1L) family, such as THESEUS1 (THE1) and FERONIA (FER) [61]. Previous research has shown the essential role of THE1 in mediating the response to cellulose impairment and suggested its function as a cell wall damage sensor [17], [62]. Several mutant lines for THE1 have been reported to date. The THE1 loss of function mutant (*the1-1*) is almost insensitive to treatment with the cellulose biosynthesis inhibitor isoxaben (ISX), while *the1-4* is a gain of function allele, lacking the intracellular kinase domain, which displays higher ISX susceptibility [17], highlighting its essential function in sensing CWI changes. In our attempt to better understand the role of CrRLK1Ls during abiotic stress and in particular in regulating salt stress responses, a collection of mutants previously described for their role in CWI maintenance has been characterized. Amongst them, we included the double *herk1the1-4* mutants originally reported lacking the function of both the CrRLK1Ls THE1 and HERKULES1 and only later characterized for being a combination of *herk1* loss of function and *the1-4* [34]. These double mutants share similar stunted growth phenotypes with *fer-4* adult plants, while loss of function mutations for HERK1, or THE1-4 show a wild type-like physiology in terms of cell elongation and rosette leaf shape [33]. In our experimental conditions, we observed that *herk1-the1-4* mutant lines not only shared similar vegetative growth but also displayed a *fer-4*-like enhanced salt sensitivity. It is interesting to notice that in the presence of salt even if *the1-1* showed no significant differences when compared to the wild type controls (as also reported in [22]), the single *the1-4* gain of function allele also showed mildly enhanced salt induced responses. The *herk1the1-4* double mutants were even more disturbed than the corresponding single mutants suggesting that combinations between these two CrRLK1Ls is required for mimicking *fer-4* in salt and non-salt-induced phenotypes.

To date, potential transient hetero-oligomerization of multiple CrRLK1Ls has been suggested, for example in the case of HERK1/ANJEA or ANX/BUPS in pollen tubes [reviewed in [12]], thus a possible interaction between HERK1/THE1-4 regulating the activity of FER cannot be excluded. FER has been reported to function as scaffold and regulate the interaction with other plasma membrane localized proteins being responsible for modulating immune responses and suggesting a complex role for FER as a signaling hub intersecting several pathways and playing multiple functions in regulating plant growth/development and reproduction [63]–[65]. *fer-4* mutants exhibit significant growth defects and their root cells burst in response to salt application [19], but to date no available data have connected other CrRLK1Ls, besides FER, to salt-induced responses. The observed phenotypes have been connected with the hypothesis that FER directly senses cell wall pectin perturbations proposed to likely happen when Na^+ ions interfere with pectin crosslinks [19] and FER's malectin domain has been suggested to bind specifically de-methyl esterified pectin domains [66]. Yet, all the available information is from *in vitro* interactions. Similarly, in yeast, the extracellular domain of the cell wall integrity WSC1 receptor, required to activate responses triggered by plasma membrane/cell wall displacement during osmotic stress, can bind to structural elements of yeast cell walls. WSC1, which is connected to the perception of mechanical stress and not to polysaccharide perception, functions as a nanospring involved in sensing the osmo-dependent cell wall/plasma membrane interphase modification upon cell shrinking or swelling [67], [68]. In plants, THE1 has been hypothesized to function in a similar manner as the yeast WSC1, being required to activate responses induced by cellulose

impairment-induced cell swelling. Interestingly ISX-induced responses could be alleviated by osmotic treatments which can rescue the cell wall/plasma membrane displacement to the control conditions [69].

Here, we report that changes in cell wall elasticity observed in response to salt [19] might be the result of modifications in the degree of methyl-esterification of homogalacturonan, since wt seedlings treated with salt showed enhanced de-methyl esterified epitopes on pectin when compared to the mock controls. It has been previously suggested that Na^+ ions can intercalate in the de-methyl esterified pectin limiting calcium mediated pectin crosslinks. Therefore, excessive calcium ions can counteract the effect of sodium rescuing the right balance of crosslinked pectins [19]. However, in our experiments we have observed that only in the presence of salt the reduction in methyl esters becomes visible, likely suggesting that salt might enhance the activity of enzymes required for the establishment of the pectin moieties. In our study we were able to detect cell wall changes at the whole seedling levels only after 24 h, but we hypothesize that small local (non-detectable) cell wall changes might happen immediately after salt application since salt-induced responses were alleviated by exogenous application of chemicals that alter pectin structure [calcium chloride and the pectin methyl esterase (PME) inhibitor ECGC [53], [64]]. This suggests that the local salt-induced pectin de-methyl esterification is sensed/perceived via the cell wall sensors FER alone or the combination of HERK1/THE, being in turn responsible for activating several main intracellular signaling pathways in plants. We also observed that while exogenous calcium can mitigate the salt-dependent HG de-methyl esterification as well as salt-triggered immediate/early responses it cannot alter the sodium specific root halotropic responses. These results are in accordance with [31], where external calcium application did not alter root salt avoidance. Inhibition of pectin modification by salt on the other hand, not only rescued early responses to salt but also halotropism was mitigated especially in the salt sensitive *fer-4* and *herkthe1-4* mutant lines. Whether calcium application can counteract salt induced responses by inhibiting the salt dependent PME activation could be hypothesized. Experiments performed to enhance tomato shelf-life, showed that fruits treated with $CaCl_2$ show less PME activity and have enhanced tissue firmness [70].

In literature the role of PMEs and PME inhibitors (PMEIs) in regulating salt stress responses is far from being understood. For example, it has been reported that reduced expression of *PMEI10* (*AT1G62760*) led to reduced salt sensitivity, likely suggesting that at least PMEI10 might function as negative regulator of salt stress responses [[71], also reviewed in [72]]. On the other hand, overexpression of *PMEI13* led to enhanced salt tolerance [73] fitting with our current observations, where PME mediated- salt-induced HG de-methyl esterification is required to activate at least in part salt-dependent signaling responses. This hypothesis is supported by our observations that salt induced cell wall damage-triggered responses are alleviated by chemicals that function in regulating pectin network organization, especially in *fer-4*/*herk1the1-4* mutants. Thus, a model could be hypothesized were salt induces pectin de-methylation through PMEs, which is sensed by FER and HERK1/THE.

Recent works have also shown that the elastic properties of the cell wall can be modified by auxin, triggering reduced cell wall rigidity mediated by de-methyl esterification of pectins [74]. The fact that root halotropism is independent of extracellular calcium [31] or MPK6 (this study) but dependent on PME inhibition (this study) as well as PIN-FORMED2 (PIN2) re-localization [31], suggests that the enhanced halotropic response observed in both salt-challenged *fer-4* and *herk1the1-4* mutant roots could be dependent either on different regulation of PMEs in the mutants or mis-regulation of PIN re-localization in response to salt stress. In a recent paper it has been reported that the physiological root waviness phenotypes observed in *fer-4* seedlings grown in tilted plates depends on altered PIN2 localization [75]. In addition, EGCG application seems to affect the localization and dynamics of several plasma membrane localized proteins (including PIN3 and FLS2) [76].

Conclusions

In this study we report and characterize in detail early and late salt stress-induced responses (Figure 1B). By dissecting the salt dependent signaling, we were able to propose the model presented in Figure 5. We describe that salt induced pectin de-methyl esterification in the cell wall is perceived not only by the cell wall sensor FERONIA (FER) alone but also by the HERKULES1 (HERK1)/THE1-4 gain of function combination having a similar role in salt stress related signaling. Pectin modification might be responsible for the activation of downstream responses, such as MPK6 phosphorylation which is required for the full activation of the salt-induced gene expression markers used in this paper. MPK6 inhibition was described to make plants more resistant to salt (*e.g.* enhanced root growth, reduced salt-triggered Ca^{2+} spike accumulation) [43]. In accordance with literature, we show that MPK6 negatively regulates at least part of the salt stress responses (salt induced gene expression) which could affect salt tolerance. The higher MPK6 phosphorylation levels detected in the salt sensitive *fer-4* and *herk1the1-4* mutants, leads to higher salt induced gene expression and a higher seedling sensitivity, suggesting that FER alone or the HERK1/THE1-4 combination is required for negatively regulating this branch of the salt induced signaling pathway. The shared oversensitive salt stress responses might suggest that the HERK1/THE1-4 combination could be required to regulate FER function. However, while calcium application alleviates early salt responses in an MPK6-dependent manner, PME inhibitor treatment can suppress also the root halotropic bending observed in the over sensitive *fer-4* and *herk1the1-4* mutants. It remains to be understood why the effects of calcium or EGCG application on regulating salt induced pectin de-methyl esterification seem to be very similar. Nonetheless, we report that also salt-induced halotropic response is altered in plants lacking the functionality of the sensors analyzed in this paper, in a pectin de-methyl esterification dependent but calcium independent manner. Further investigations will be required to understand the molecular mode of action and the role of CrRLK1Ls in detecting cell wall changes induced upon salt stress, which are responsible for a major part of stress responses in Arabidopsis.

Materials and methods

Plant materials and growth condition

Arabidopsis thaliana ecotype Col-0 was used as wildtype. Experiments were conducted on several mutants listed in Table S1. Before sowing, seeds were surface sterilized with 50% bleach for 10 min and washed 3 times with sterile distilled MilliQ water. The seeds were sown after two days of vernalization at 4°C in the dark. For MPK6 phosphorylation assays and gene expression analysis 15 seeds were germinated and grown in 2 mL half-strength MS medium supplemented with 0.1% 2(N-morpholino) ethane sulphonic acid (MES) buffer and 0.5% sucrose (pH 5.7) in 12 well multi-well plates. Chemicals used for the analysis of calcium effects on salt induced responses were in distilled sterile MilliQ water (used as mock), 5 mM $CaCl_2$ (in sterile water), 100 mM NaCl (in sterile water) or $CaCl_2$/NaCl combinations. Similarly, chemicals used for the analysis of PME inhibition were DMSO (mock), 50 µM EGCG (Sigma-Aldrich; in DMSO), 100 mM NaCl or a combination of EGCG/NaCl. All these experiments were performed on 7-day old seedlings. All treatments contained equal amounts of DMSO. For the halotropism assays, the media used were made with the recipe stated above and supplemented with 1% agar. Seedlings were grown in 70-degree holders and after 4 days the lower part of the media under a diagonal line was cut with sterile thin glass slide and replaced with 1/2 MS supplemented with H_2O (mock) or 200 mM NaCl. In the case of halotropism assays performed in the presence of EGCG the agar strength of the medium was enhanced to 1.25% to avoid root tip growth into the medium. In these experiments lower gradients contained DMSO (mock), 50 µM EGCG, 200 mM NaCl or a combination of EGCG/NaCl. All treatments contained equal amounts of DMSO. All results are based on independent experiments and number of biological replicates are stated in figure legends or are shown in supplemental figures. An Epson Scanner was used to image plates at 24 h or 48 h after treatment. For cell wall extraction analysis and ion content measurements seedlings were grown for 6 days in 250 mL Erlenmeyer flasks

containing 110 mL of 1/2 MS medium, 0.1% MES and 1% sucrose (pH 5.7) on a flask shaker at the constant speed of 130 rotations per minute. Treatments were performed with H_2O, or 100 mM NaCl and samplings done after 24 h. For time-lapse growth analysis seedlings were grown in 1/2 MS medium, 0.1% MES (pH 5.7) supplemented with 1% agar in square plates positioned in vertical holders (90 degrees). After 4 days, seedlings were transferred in mock (H_2O) or 125 mM NaCl containing plates and pictures taken, with an automatized system every 20 minutes for 42 h. For the beaching assays seeds were germinated and grown for 10 days in horizontally oriented square plates containing 1/2 MS medium, 0.1% MES, 1% agar (+/- 150 mM NaCl) pH 5.7. Seedlings were grown in long-day conditions (16 h light, 22°C/8 h dark, 18°C) at 150 µmol m^{-2} s^{-1} photon flux density.

Salt stress assays

Analysis of root halotropic responses was performed after scanning seedlings grown in square plates with an Epson scanner. Roots were traced with SmartRoot 2.0, an open-source plugin for Fiji/ImageJ (https://fiji.sc) and root directions were used to determine root bending (angle). For time-lapse based root assays, an automated script based on root edge detection was developed (https://github.com/jasperlamers/timelapse-backtracing) and used to detect and trace root length using the last picture taken. For salt tolerance assays, plants were grown in pots under short light photoperiod (8 h light/16 h dark) in greenhouses set at 21 ºC and with controlled humidity (70%). Ten days after germination (1-week old seedlings) plants were transferred to new pots saturated with 0 or 75 mM of NaCl solution. For 3 weeks plants were watered twice a week with MilliQ water. Rosette fresh weights were measured at the end of the 3 weeks. The experiments were repeated 2 times with similar results.

Gene expression analysis and MPK6 phosphorylation assays

7-day old seedlings grown as stated above, were treated with mock or 100 mM NaCl. After 15 minutes (MPK6 assays) or 1 h (gene expression analysis), seedlings were flash frozen in 2 mL tubes containing metal beads in liquid nitrogen and ground into a fine powder by using a paint shaker. Total RNA was isolated using EasyPrep Plant Total RNA Extraction Miniprep (Bioland). DNAse treatment were performed as in [18], [39] and cDNA synthetized with iScript cDNA Synthesis Kit (Biorad) according to the manufacturer's instructions. qRT-PCR analysis was conducted by CFX96 Real-Time System (Biorad) using qPCRBIO SyGreen Blue Mix Lo-ROX (Sopachem) master mix and primers (Table S2) diluted according. Gene expression analyses were performed as in [17], [18] and are shown as relative expression to *ACT2*, used as reference gene. For protein extraction, the following extraction buffer was used; 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 0.1% (v/v) Tween 20, 1 mM PMSF, 1 mM DTT, 1× protease inhibitor cocktail (Sigma, P9599), 1x phosphatase inhibitor (Sigma, P2850). After 15 min incubation in ice, samples were centrifuged at 12000 rpm at 4°C for 15 min. The supernatants were used for western blot analysis. Protein concentration was assayed by Bradford assay (Biorad). 15 µg of total proteins were loaded on 10% SDS-PAGE gel. The samples were mixed with 4× loading buffer (240 mM Tris pH 6.8, 8% (w/v) SDS, 40% glycerol, 5% β-mercaptoethanol, 0.04% (w/v) bromophenol blue) and heated for 2 min at 95°C. Protein samples were separated and then transferred to the nitrocellulose membrane in Trans-Blot Turbo Transfer System (Biorad) at 25 V, 1.3 A for 7 min. After blocking with Tris Buffered Saline with Tween 20 (TBST) containing 5% (w/v) bovine serum albumin (BSA) (Sigma, A3608) for 1 h, the membrane was incubated overnight with the primary phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (Cell Signaling Technology, #9101). Subsequently, the membrane was washed 3 times with TBST and then incubated with secondary antibody, anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology, #7074) for 1 h. After washing 3 times with TBST the chemiluminescent was conducted by adding Clarity Western ECL Substrate (Biorad, #1705061) for 2 min and detected by ChemiDoc imaging system (Biorad). For equal loading analysis, membranes were stripped by stripping

solution (100 mM Tris at pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) for 1 h, and blocked as described previously incubation with β-Actin (C4) antibody (Santa Cruz biotechnology, sc-47778) for 1 h. Detection of chemiluminescence was performed as mentioned before.

Cell wall extraction and dot blot assays

After 24 h treatment (see above for details) seedlings were snap frozen in liquid nitrogen and ground into a fine powder with a paint shaker. At least 3 biological replicates for each treatment were used. Cell wall material (AIR) was extracted as in [77]. After an overnight of drying, AIRs were weighed and sterile distilled MilliQ water (ph 7.2), used to extract pectins. Approximately 2- to 3 mg of AIR were boiled with 250 µl water for 1 h. After a short centrifuge (15 min, 13.000xg), supernatants were collected, and samples boiled again for an additional hour with 250 µl water. After 1h supernatants deriving from the same samples were pooled. Total sugar content was measured with the phenol/H_2SO_4 based method (Dubois) using galacturonic acid as standard [47]. Prior spotting on nitrocellulose membrane, 5 µg total sugars were adjusted to 5 µl by adding (if necessary) distilled MilliQ water (ph 7.2). For positive and negative control, the same amount of sugars were incubated for 1 h with $NaCO_3$ pH 11 to remove pectin methyl esterification. Membranes were dried overnight at room temperature. Blocking (5% milk in PBS) was performed for 1 h. Next, membranes were incubated for 1 h with primary antibodies [LM20, 19 and 2F4, (http://www.plantprobes.net/index.php)] in the following buffers/concentrations; 1:250 in 5% milk powder in 1 × PBS for both Lm20/19 and 1:250 in 5% milk in T/Ca/S buffer (Tris-HCl 20 mM pH 8.2, CaCl2 0.5 mM, NaCl 150 mM) (http://www.plantprobes.net/index.php) [50]. After PBST washes (3 times) membranes were incubated with HRP-conjugated secondary antibodies (rat (IgM)/LM20 and LM19 mouse (IgG)/2F4 (in 5% milk PBS) and after washing chemiluminescence induced with Clarity Western ECL Substrate (Biorad, #1705061) and detected with ChemiDoc imaging system (Biorad).

Statistical analysis

IBM SPSS Statistics v24 was used to perform statistical significance (Student's t-test, ANOVA). Statistically significant differences are indicated with different letters for the one-way ANOVA/Tukey's HSD test at $\alpha=0.05$. Pairwise comparisons, and statistically significant differences according to Student's t-test are represented with *P<0.05, **P<0.01, ***P<0.001. For root time-lapse growth analysis, root elongation rate was calculated over 2 h intervals, averaged per hour and expressed as a ratio between salt and control treatments. Each curve comprises several dots, representing the average growth rate and the corresponding standard error analyzed in each of the time lapse images containing 8 seedlings per plates (only correctly segmented roots were included, n>12). In this assay statistical comparisons were performed by analyzing every timepoints for equal variance using a Levene's test, followed by t-test to compare different genotypes at the same timepoint. The significance level (p=0.05) was corrected for multiple testing by using step-wise Sidak correction.

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Author Contributions

N.G.B conceived the presented idea and performed most of the experiments of the study. C.T and N.G.B have designed the work. E.VZ provided the time-lapse based root growth measurements and the statistical analysis associated to that. J.

L. developed the automated software to analyze images from the time-lapse setup and assisted with halotropism assays. W.H performed the halotropism assays with salt and EGCG combinations. C.T supervised the findings and discussed the results with N.G.B., together they wrote the manuscript, with help of all authors.

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Figure Legends

Figure 1. *herk1the1-4* and *fer-4* mutant lines show enhanced early responses to salt stress. A. Cotyledon bleaching analysis was conducted in seedlings grown for 10 days in the presence of 150 mM NaCl. Values, expressed in percentage are the average of 4 independent experiments. Bars represent Standard Error (SE). One-way ANOVA and Tukey's HSD (α= 0.05) were used to compare differences between genotypes within one treatment condition (salt). **B.** Timeline of the treatments performed in this paper together with the terminology used. **C.** 7-day old seedlings of Col-0, *fer-4* and *herk1the1-4* were grown in liquid 0.5% MS and treated for 15 minutes with H_20 (mock, m) or 100 mM NaCl (n). Phosphorylation. of MPKs was detected by immunoblot assay using specific antibody against the pMPK36 and pMPK3 (α-p44/42-ERK). Equal loading performed upon stripping was carried out by using anti β-actin Arabidopsis antibody. Similar results were obtained in 3 biological replicates (n=3). **D.** Seedlings as in **C** were treated for 1h to analyze the expression levels of selected salt-induced marker genes. Expression of *ProPEP3*, *WRKY40, RRTF1* relative to *ACT2,* determined by qRT-PCR is represented in the histograms as the average of 3 biological replicates (n=3). Bars indicate standard deviation (SD). Statistical comparisons have been made by using One-way ANOVA and Tukey's HSD (α= 0.05) considering the 3 different genotypes within the same treatment (black letters for mock comparisons, red for NaCl treatments). **E.** 7-day old seedlings of Col-0, *mpk6-3* were treated as in D. and gene expression analysis was performed after 1h treatment H_20 (mock, m) or 100 mM NaCl. n=3. Bars indicate standard deviation (SD). Asterisks show statistical comparisons between NaCl-treated Col-0 and NaCl-treated *mpk6-3* according to Student's t test, ***P < 0.001).

Figure 2. *herk1the1-4* and *fer-4* mutant lines show enhanced late responses to salt stress. A. 4-day old seedlings of Col-0, *fer-4* and *herk1the1-4* were subjected to NaCl gradient (for detailed explanation see Materials and Methods section) and root angle was analyzed with Smartroot 2.0 after 24 h of treatment. Histogram represents the average root angle of 3 independent experiments, while bars show the average of standard errors (SE). Bars represent Standard Error (SE). One-way ANOVA and Tukey's HSD (α= 0.05) were used to compare differences between genotypes within one treatment condition. Black letters show the comparisons between different genotypes in mock conditions while the red ones show those performed in the presence of NaCl. (n=70). **B.** 4-day old seedlings of Col-0, *mpk6-3* were treated as in A and root angle was analyzed after 24 h and 48 h. Histogram represents the average root angle of 2 independent experiments, while bars show the standard errors (SE). (n=45). Asterisks show statistical comparisons between the same treatment in different genotypes; ***P < 0.001, *n.s.* not significant. **C.** Growth rate and root elongation kinetic was monitored for 42 h in a time lapsed growth chamber settings in 4-day old seedlings of Col-0, *fer-4* (left panel) or Col-0 and *herk1the1-4* (right panel) transferred at time 0 h to new plates containing mock or 125 mM NaCl. Root elongation rate was calculated over 2 h intervals, averaged per hour and expressed as a ratio between salt and control treatments. Each of the curve dots represent the average length and the corresponding standard error analyzed in each of the time lapse images containing 5 seedlings per plates (n=15). Experiments were repeated twice with similar results. Statistical comparisons were performed by analyzing every timepoints for equal variance using a Levene's test, followed by t-test to compare different genotypes at the same timepoint. The significance level (p=0.05) was corrected for multiple testing by using step-wise Sidak correction (* = significant).

Figure 3. Salt-induced responses are alleviated by $CaCl_2$ application A. Root Growth rate elongation monitored for 42 h in 4-day old seedlings of Col-0 treated from time 0 h (transferring time) with mock, 125 mM NaCl, 5 mM $CaCl_2$ or a combination of NaCl and $CaCl_2$ (NaCl+ $CaCl_2$). Root growth rate is expressed as a ratio between NaCl and control treatments or NaCl+$CaCl_2$ and control. Each of the dots represent the average length and the corresponding standard error analyzed in n=15 seedlings per treatment. Statistical comparisons were performed by analyzing every timepoints for equal

variance using a Levene's test, followed by t-test to compare different genotypes at the same timepoint. Samples were corrected for multiple testing by using Sidak correction. *, P< 0.05 **B.** 6-day old seedlings of Col-0, *fer-4* and *herk1the1-4* were grown in liquid 0.5%MS and treated for 15 minutes with H_20 (mock, m), 100 mM NaCl (N), 5 mM $CaCl_2$ or a combination of NaCl and $CaCl_2$(NC). MPKs phosphorylation detected by immunoblot assay, was performed by using specific antibody against the phosphorylated forms of both MPK3 and MPK6 (α-p44/42-ERK). Equal loading was carried out by using anti β-actin Arabidopsis antibody. Similar results are shown in figure S2. **C.** Expression of *ProPEP3*, *WRKY40, RRTF1* determined by qRT-PCR and relative to *ACT2,* was performed in 6-day old seedlings of Col-0, fer-4, *herk1the1-4* treated as in B. for 1h. n=3. Bars indicate standard deviation (SD). One-way ANOVA and Tukey's HSD (α= 0.05) was used to analyze statistical differences between treatments within the same genotypes.

Figure 4. Salt induced alteration methylation of loosely bound pectins can be alleviated by $CaCl_2$ or EGCG application. A. Six-day old Arabidopsis wild type seedlings were grown in flasks and treated for 24 h with H_20 (m) or 100 mM NaCl (N). 3 biological replicates, containing 50 seedlings each, for mock and NaCl were used to extract the Alcohol Insoluble Residues (AIRs). Pectin factions were obtained by boiling the AIRs with distilled sterile water (pH 7.2) as described in materials and methods. The extracted sugars were quantified and 5 μg of total sugars were transferred in tubes containing the same volume of ddH_2O (native) or 0.1M final concentration of Na_2CO_3 pH 11, to remove methyl esterification [demethylated HG (labeled with deM-HG)]. After 1 h incubation, sugars were spotted on nitrocellulose membranes. Dot-blot were performed with 2F4 (in T/C/N buffer) or LM20 and LM19 (in PBS+BSA buffers). **B.** Six-day old seedlings of Col-0, *fer-4* and *herk1the1-4* mutants were grown as in A. Sugars were extracted as in A and 5 μg spotted on nitrocellulose membranes after 1h incubation with ddH_2O (native) or Na_2CO_3 pH 11 (deM-HG). Dot-blot was performed with 2F4**. C.** 6-day old seedlings of Col-0, *fer-4* and *herk1the1-4* were treated for 15 min with DMSO (D), 100 mM NaCl (N), 50 μM EGCG (E) and NaCl/EGCG (NE). 15 μg of total proteins loaded and phosphorylation of MPK6 analyzed via western blot assays with α p42/44 antibody. β-actin and ponceau red are shown for equal loading. **D.** Expression of *ProPEP3*, *WRKY40, RRTF1* determined by qRT-PCR and relative to *ACT2,* was performed in 6-day old seedlings of Col-0, *fer-4*, *herk1the1-4* treated for 1 h with DMSO (mock), 100 mM NaCl, 50 μM EGCG and NaCl/EGCG. n=3. Bars indicate standard deviation (SD). Statistical comparisons have been made by using One-way ANOVA and Tukey's HSD (α= 0.05) considering the 3 different genotypes within the same treatment (black letters for mock comparisons, red for NaCl treatments). **E.** 4-day old seedlings of Col-0, *fer-4* and *herk1the1-4* were subjected to halotropism assay with DMSO (mock), 100 mM NaCl, 50 μM EGCG and NaCl/EGCG gradients. Root angle was after analyzed after 24 h, 48 h and 72 h. Histogram represents the average root angle of 3 independent experiments, while bars show the standard errors (SE). (n=75). Letters display One-way ANOVA and Tukey's HSD (α= 0.05) within the same genotype. Red capital letters represent statistically significant differences according to one-way ANOVA and Tukey's HSD (α= 0.05) between genotypes within one treatment condition. **F.** Six-day old Arabidopsis wild type seedlings were grown in flasks and treated for 24 h with DMSO (D), 100 mM NaCl (N), 50 μM EGCG (e) and NaCl/EGCG (NE). (n=3). **G.** Similarly, 3 other biological replicates, were treated with with H_20 (m) or 100 mM NaCl (N), 5 mM $CaCl_2$ (c) or a combination of NaCl and $CaCl_2$(NC). As in G 5 μg of total sugars were transferred in tubes containing the same volume of ddH2O (native) or 0.1M final concentration of Na2CO3 pH 11, to remove methyl esterification (deM-HG). Dot-blot was performed with 2F4.

Figure 5. Hypothetical model based on the findings of this study. Salt treatment alters the degree of methyl esterification of homogalacturonan causing cell wall damage induced intracellular responses. Application of exogenous calcium or PME inhibition, can alleviate cell wall alterations likely mitigating intracellular signals. The cell wall sensor

FERONIA (FER) is responsible for attenuating most of the salt dependent phenotypes is mimicked by HERKULES1 (HERK1) and a truncated version of THE1 (namely THE1-4). We hypothesize that the cell wall sensors HERK1 and THE1-4 might interact together to maintain FER functionality. Salt induced pectin modification has been observed in the wt as well as in these mutants suggesting that these sensors might be only functioning as antennas to perceive the damage. Interestingly, salt-induced root skewing seems to be altered in these mutants (red inhibitor arrow), independently of the MPK6 cascade which is instead required for the full expression of salt induced marker genes. The regulation of salt dependent root bending which requires PIN2 re-localization can be alleviated by PME inhibition but not by calcium application

Figures

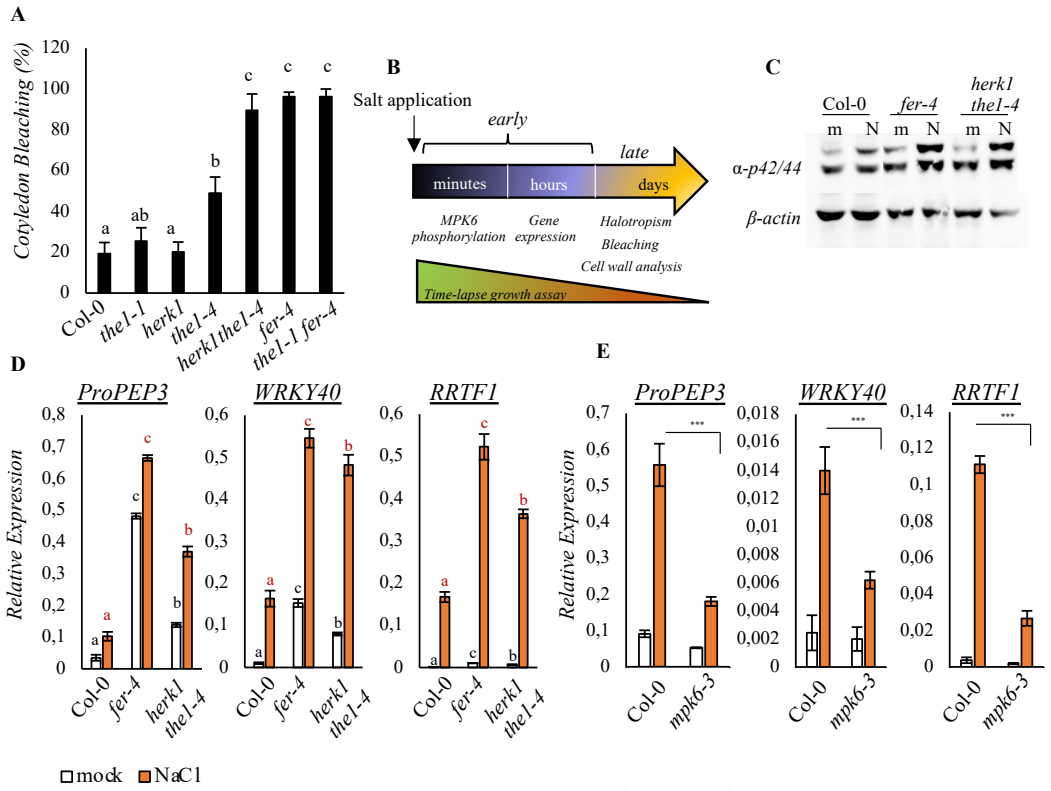

Figure 1. *herk1the1-4* and *fer-4* mutant lines show enhanced early responses to salt stress. A. Cotyledon bleaching analysis was conducted in seedlings grown for 10 days in the presence of 150 mM NaCl. Values, expressed in percentage are the average of 4 independent experiments. Bars represent Standard Error (SE). One-way ANOVA and Tukey's HSD (α= 0.05) were used to compare differences between genotypes within one treatment condition (salt). **B.** Timeline of the treatments performed in this paper together with the terminology used. **C.** 7-day old seedlings of Col-0, *fer-4* and *herk1the1-4* were grown in liquid 0.5% MS and treated for 15 minutes with H_20 (mock, m) or 100 mM NaCl (n). Phosphorylation. of MPKs was detected by immunoblot assay using specific antibody against the pMPK36 and pMPK3 (α-p44/42-ERK). Equal loading performed upon stripping was carried out by using anti β-actin Arabidopsis antibody. Similar results were obtained in 3 biological replicates (n=3). **D.** Seedlings as in **C** were treated for 1h to analyze the expression levels of selected salt-induced marker genes. Expression of *ProPEP3*, *WRKY40, RRTF1* relative to *ACT2,* determined by qRT-PCR is represented in the histograms as the average of 3 biological replicates (n=3). Bars indicate standard deviation (SD). Statistical comparisons have been made by using One-way ANOVA and Tukey's HSD (α= 0.05) considering the 3 different genotypes within the same treatment (black letters for mock comparisons, red for NaCl treatments). **E.** 7-day old seedlings of Col-0, *mpk6-3* were treated as in D. and gene expression analysis was performed after 1h treatment H_20 (mock, m) or 100 mM NaCl. n=3. Bars indicate standard deviation (SD). Asterisks show statistical comparisons between NaCl-treated Col-0 and NaCl-treated *mpk6-3* according to Student's t test, ***P < 0.001).

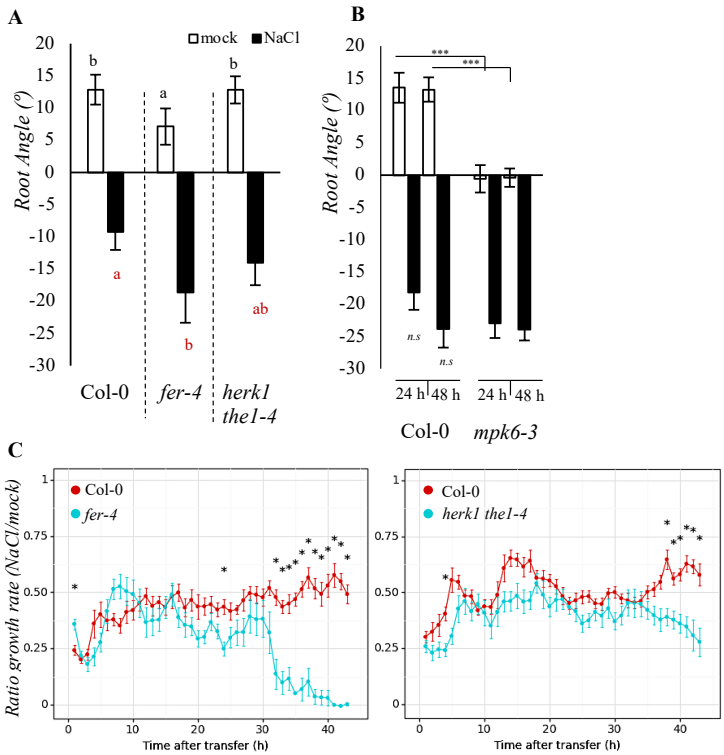

Figure 2. *herk1the1-4* and *fer-4* mutant lines show enhanced late responses to salt stress. A. 4-day old seedlings of Col-0, *fer-4* and *herk1the1-4* were subjected to NaCl gradient (for detailed explanation see Materials and Methods section) and root angle was analyzed with Smartroot 2.0 after 24 h of treatment. Histogram represents the average root angle of 3 independent experiments, while bars show the average of standard errors (SE). Bars represent Standard Error (SE). One-way ANOVA and Tukey's HSD (α= 0.05) were used to compare differences between genotypes within one treatment condition. Black letters show the comparisons between different genotypes in mock conditions while the red ones show those performed in the presence of NaCl. (n=70). **B.** 4-day old seedlings of Col-0, *mpk6-3* were treated as in A and root angle was analyzed after 24 h and 48 h. Histogram represents the average root angle of 2 independent experiments, while bars show the standard errors (SE). (n=45). Asterisks show statistical comparisons between the same treatment in different genotypes; ***P < 0.001, *n.s.* not significant. **C.** Growth rate and root elongation kinetic was monitored for 42 h in a time lapsed growth chamber settings in 4-day old seedlings of Col-0, *fer-4* (left panel) or Col-0 and *herk1the1-4* (right panel) transferred at time 0 h to new plates containing mock or 125 mM NaCl. Root elongation rate was calculated over 2 h intervals, averaged per hour and expressed as a ratio between salt and control treatments. Each of the curve dots represent the average length and the corresponding standard error analyzed in each of the time lapse images containing 5 seedlings per plates (n=15). Experiments were repeated twice with similar results. Statistical comparisons were performed by analyzing every timepoints for equal variance using a Levene's test, followed by t-test to compare different genotypes at the same timepoint. The significance level (p=0.05) was corrected for multiple testing by using step-wise Sidak correction (* = significant).

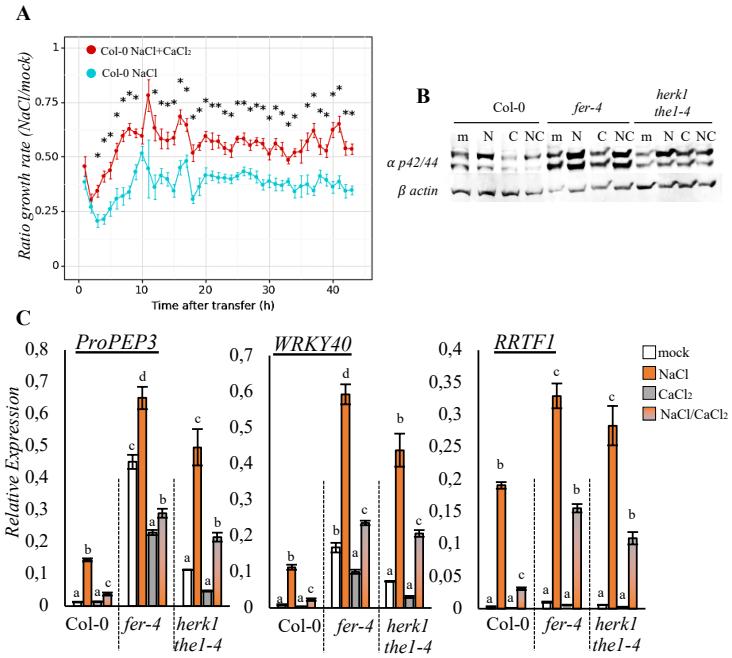

Figure 3. Salt-induced responses are alleviated by $CaCl_2$ application A. Root Growth rate elongation monitored for 42 h in 4-day old seedlings of Col-0 treated from time 0 h (transferring time) with mock, 125 mM NaCl, 5 mM $CaCl_2$ or a combination of NaCl and $CaCl_2$ (NaCl+ $CaCl_2$). Root growth rate is expressed as a ratio between NaCl and control treatments or NaCl+$CaCl_2$ and control. Each of the dots represent the average length and the corresponding standard error analyzed in n=15 seedlings per treatment. Statistical comparisons were performed by analyzing every timepoints for equal variance using a Levene's test, followed by t-test to compare different genotypes at the same timepoint. Samples were corrected for multiple testing by using Sidak correction. *, P< 0.05 **B.** 6-day old seedlings of Col-0, *fer-4* and *herk1the1-4* were grown in liquid 0.5%MS and treated for 15 minutes with H_20 (mock, m), 100 mM NaCl (N), 5 mM $CaCl_2$ or a combination of NaCl and $CaCl_2$(NC). MPKs phosphorylation detected by immunoblot assay, was performed by using specific antibody against the phosphorylated forms of both MPK3 and MPK6 (α-p44/42-ERK). Equal loading was carried out by using anti *β*-actin Arabidopsis antibody. Similar results are shown in figure S2. **C.** Expression of *ProPEP3*, *WRKY40, RRTF1* determined by qRT-PCR and relative to *ACT2,* was performed in 6-day old seedlings of Col-0, fer-4, *herk1the1-4* treated as in B. for 1h. n=3. Bars indicate standard deviation (SD). One-way ANOVA and Tukey's HSD (*α*= 0.05) was used to analyze statistical differences between treatments within the same genotypes.

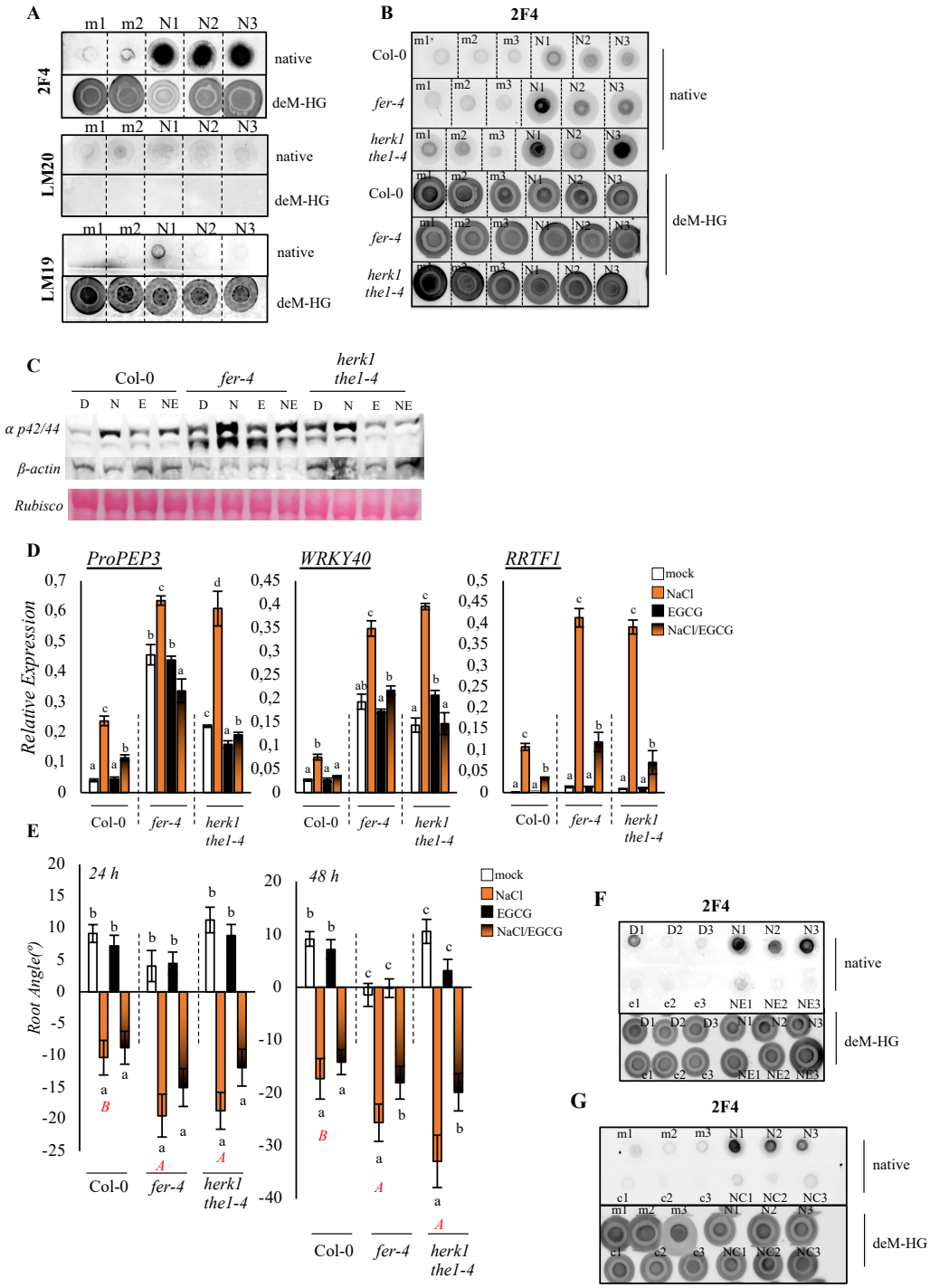
A
m1 m2 N1 N2 N3
2F4
LM20
LM19
native
deM-HG
B
2F4
Col-0
fer-4
herk1 the1-4
native
deM-HG
C
Col-0
fer-4
herk1 the1-4
D N E NE
α p42/44
β-actin
Rubisco
D
ProPEP3
WRKY40
RRTF1
Relative Expression
mock
NaCl
EGCG
NaCl/EGCG
E
24 h
48 h
Root Angle(°)
F
2F4
D1 D2 D3 N1 N2 N3
e1 e2 e3 NE1 NE2 NE3
native
deM-HG
G
2F4
m1 m2 m3 N1 N2 N3
c1 c2 c3 NC1 NC2 NC3
native
deM-HG

Figure 4. Salt induced alteration methylation of loosely bound pectins can be alleviated by $CaCl_2$ or EGCG application. A. Six-day old Arabidopsis wild type seedlings were grown in flasks and treated for 24 h with H_20 (m) or 100 mM NaCl (N). 3 biological replicates, containing 50 seedlings each, for mock and NaCl were used to extract the Alcohol Insoluble Residues (AIRs). Pectin factions were obtained by boiling the AIRs with distilled sterile water (pH 7.2) as described in materials and methods. The extracted sugars were quantified and 5 μg of total sugars were transferred in tubes containing the same volume of ddH_2O (native) or 0.1M final concentration of Na_2CO_3 pH 11, to remove methyl esterification [demethylated HG (labeled with deM-HG)]. After 1 h incubation, sugars were spotted on nitrocellulose membranes. Dot-blot were performed with 2F4 (in T/C/N buffer) or LM20 and LM19 (in PBS+BSA buffers). **B.** Six-day old seedlings of Col-0, *fer-4* and *herk1the1-4* mutants were grown as in A. Sugars were extracted as in A and 5 μg spotted on nitrocellulose membranes after 1h incubation with ddH_2O (native) or Na_2CO_3 pH 11 (deM-HG). Dot-blot was performed with 2F4**. C.** 6-day old seedlings of Col-0, *fer-4* and *herk1the1-4* were treated for 15 min with DMSO (D), 100 mM NaCl (N), 50 μM EGCG (E) and NaCl/EGCG (NE). 15 μg of total proteins loaded and phosphorylation of MPK6 analyzed via western blot assays with α p42/44 antibody. β-actin and ponceau red are shown for equal loading. **D.** Expression of *ProPEP3*, *WRKY40, RRTF1* determined by qRT-PCR and relative to *ACT2,* was performed in 6-day old seedlings of Col-0, *fer-4*, *herk1the1-4* treated for 1 h with DMSO (mock), 100 mM NaCl, 50 μM EGCG and NaCl/EGCG. n=3. Bars indicate standard deviation (SD). Statistical comparisons have been made by using One-way ANOVA and Tukey's HSD (α= 0.05) considering the 3 different genotypes within the same treatment (black letters for mock comparisons, red for NaCl treatments). **E.** 4-day old seedlings of Col-0, *fer-4* and *herk1the1-4* were subjected to halotropism assay with DMSO (mock), 100 mM NaCl, 50 μM EGCG and NaCl/EGCG gradients. Root angle was after analyzed after 24 h, 48 h and 72 h. Histogram represents the average root angle of 3 independent experiments, while bars show the standard errors (SE). (n=75). Letters display One-way ANOVA and Tukey's HSD (α= 0.05) within the same genotype. Red capital letters represent statistically significant differences according to one-way ANOVA and Tukey's HSD (α= 0.05) between genotypes within one treatment condition. **F.** Six-day old Arabidopsis wild type seedlings were grown in flasks and treated for 24 h with DMSO (D), 100 mM NaCl (N), 50 μM EGCG (e) and NaCl/EGCG (NE). (n=3). **G.** Similarly, 3 other biological replicates, were treated with with H_20 (m) or 100 mM NaCl (N), 5 mM $CaCl_2$ (c) or a combination of NaCl and $CaCl_2$(NC). As in G 5 μg of total sugars were transferred in tubes containing the same volume of ddH2O (native) or 0.1M final concentration of Na2CO3 pH 11, to remove methyl esterification (deM-HG). Dot-blot was performed with 2F4.

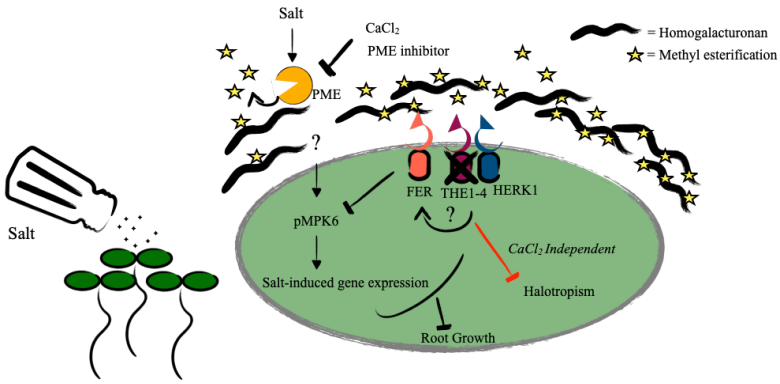

Figure 5. Hypothetical model based on the findings of this study. Salt treatment alters the degree of methyl esterification of homogalacturonan causing cell wall damage induced intracellular responses. Application of exogenous calcium or PME inhibition, can alleviate cell wall alterations likely mitigating intracellular signals. The cell wall sensor FERONIA (FER) is responsible for attenuating most of the salt dependent phenotypes is mimicked by HERKULES1 (HERK1) and a truncated version of THE1 (namely THE1-4). We hypothesize that the cell wall sensors HERK1 and THE1-4 might interact together to maintain FER functionality. Salt induced pectin modification has been observed in the wt as well as in these mutants suggesting that these sensors might be only functioning as antennas to perceive the damage. Interestingly, salt-induced root skewing seems to be altered in these mutants (red inhibitor arrow), independently of the MPK6 cascade which is instead required for the full expression of salt induced marker genes. The regulation of salt dependent root bending which requires PIN2 re-localization can be alleviated by PME inhibition but not by calcium application