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ORIGINAL ARTICLE

The root growth reduction in response to mechanical stress involves ethylene-mediated microtubule reorganization and transmembrane receptor-mediated signal transduction in *Arabidopsis*

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Abstract

Key message **We found that mutations in a Ca^{2+}-permeable mechanosensitive channel MCA1, an ethylene-regulated microtubule-associated protein WDL5, and a versatile co-receptor BAK1 affect root growth response to mechanical stress.**

Abstract Plant root tips exposed to mechanical impedance show a temporal reduction in the elongation growth. The process involves a transient Ca^{2+} increase in the cytoplasm followed by ethylene signaling. To dissect the molecular mechanisms underlying this response, we examined the root growth of a series of Arabidopsis mutants with potentially altered response to mechanical stress after transfer from vertical to horizontal plates that were covered by dialysis membrane as an impedance. Among the plant hormone-response mutants tested, the ethylene-insensitive mutant *ein3* was confirmed to show no growth reduction after the transfer. The root growth reduction was attenuated in a mutant of *MCA1* encoding a Ca^{2+}-permeable mechanosensitive channel and that of *WDL5* encoding an ethylene-regulated microtubule-associated protein. We also found that the growth reduction was enhanced in a mutant of *BAK1* encoding a co-receptor that pairs with numerous leucine-rich repeat receptor kinases to modulate growth and immunity. These results suggest the root growth reduction in response to mechanical stress involves ethylene-mediated microtubule reorganization and also transmembrane receptor-mediated signal transduction.

Keywords Arabidopsis · Calcium channel · Ethylene · Mechanical impedance · Microtubule

Introduction

During soil penetration, plant roots sense and respond to gravity and the subsequent mechanical stress generated by their gravitropic growth against obstacles while they need to cope with other environmental conditions such as nutrients, water availability, and soil microbes. The mechanically impeded roots show a reduction in the elongation rate, an increase in the root diameter, and altered patterns of lateral root initiation (Bengough and Mullins 1990). A detailed study using Arabidopsis seedlings has shown that once the root cap receives mechanical stimulation, it down-regulates gravitropism, allowing the formation of a new tropic response (Massa and Gilroy 2003). The first step of the response of root tips to mechanical barriers is a transient increase of Ca^{2+} ions in the cytoplasm. *MID1-COMPLEMENTING ACTIVITY1* (*MCA1*) and its paralogous *MCA2* are suggested to encode a component of mechanosensitive Ca^{2+} channel complexes (Nakagawa et al. 2007). The *mca1*

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mutant root has a reduced ability to penetrate hard agar from soft agar, revealing a role of MCA1 in overcoming mechanical barriers (Nakagawa et al. 2007; Yamanaka et al. 2010). Cytoplasmic Ca^{2+} ions in turn trigger a variety of secondary Ca^{2+}-dependent responses including modulation of enzyme activity, induction of gene expression, production of reactive oxygen species, and activation of ethylene signaling. These collectively allow plant roots to circumvent physical obstacles and grow downward (Monshausen and Gilroy 2009; Kurusu et al. 2013). However, the exact nature of the molecular events and their components from mechano-sensing to growth response are yet to be fully understood.

In Arabidopsis, ethylene signaling is mediated by endoplasmic reticulum-located ETHYLENE-INSENSITIVE2 (EIN2) whose cleaved product shuttles into the nucleus to activate key transcription factors EIN3 and EIN3-LIKE1 (EIL1) (Ju et al. 2012). In hypocotyl growth, EIN3/EIL1 activate *PHYTOCHROME INTERACTING FACTOR3* (*PIF3*) and *ETHYLENE RESPONSE FACTOR1* (*ERF1*), which promote hypocotyl elongation in the light and inhibit it in the dark, respectively (Zhong et al. 2012). ERF1 integrates signals from jasmonate (JA) and ethylene during defense response (Lorenzo et al. 2003), while JA signaling is involved in touch-induced growth alterations in the shoot (Chehab et al. 2012). Another EIN3 target gene, *WAVE-DAMPENED5* (*WDL5*), has been shown to act in ethylene-inhibited hypocotyl elongation in the dark. WDL5 binds to cortical microtubules and regulates microtubule reorientation (Sun et al. 2015; Ma et al. 2016). Involvement of these factors in the response to mechanical stress in the root remains to be addressed.

The mechanical sensing may also involve several classes of receptor-like kinases (RLKs) (Hamant and Haswell 2017). FERONIA (FER), a member of the CrRLK1L (*Catharanthus roseus* RLK1-like) family in Arabidopsis recognizing rapid alkalinization factor (RALF) peptides, RALF1, RALF17, and RALF23, monitors cell wall integrity and plays a role in cytoplasmic Ca^{2+} homeostasis, immune signaling, and ROS production (Haruta et al. 2014; Li et al. 2015; Stegmann et al. 2017; Feng et al. 2018). The *fer* mutant is hypersensitive to ethylene and exhibits growth phenotypes consistent with impaired mechanical development, including biased root skewing, an inability to penetrate hard agar layers, and abnormal growth responses to impenetrable obstacles (Shih et al. 2014). THESEUS1 (THE1), another member of CrRLK1L, recognizes RALF34 and triggers growth inhibition and defense responses upon perturbation of the cell wall, in part, with the aid of FER (Gonneau et al. 2018). Furthermore, a versatile co-receptor BRASSINOSTEROID INSENSITIVE1 (BRI1)-ASSOCIATED KINASE1 (BAK1)/SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE3 (SERK3), which pairs with numerous leucine-rich repeat (LRR) kinases to modulate growth and immunity (Chinchilla et al. 2009; Postel et al. 2010; Yasuda et al. 2017), has been shown to mediate the RALF1-induced inhibition of root cell expansion (Dressano et al. 2017).

In a previous study, we have developed a highly sensitive assay method to detect root growth reduction by mechanical impedance using Arabidopsis seedlings. According to this method, the seedlings exhibit reduced root growth and ectopic root hair formation when those grown on vertical plates are transferred to horizontal plates covered with impenetrable dialysis membrane (Okamoto et al. 2008). Using this assay system, we identified omeprazole, a gastric proton pump inhibitor, as a strong enhancer of root growth reduction from screening a chemical library, suggesting the involvement of calcium or proton pumps in the root growth response (Okamoto et al. 2018). Aminocyclopropane carboxylate, a precursor of ethylene, also enhanced the growth reduction, while silver ions, which block ethylene perception, and salicylic acid (SA) attenuated the response (Okamoto and Takahashi 2019). To identify further components involved in the mechanical stress perception, signal transduction, or growth response in the root, we applied this assay method to examine Arabidopsis mutants with potentially altered growth response. The results not only confirm the involvement of Ca^{2+} channels and ethylene signaling but also provide evidence for the involvement of receptor kinase signaling and microtubule reorganization in the root growth response to mechanical stress.

Materials and methods

Plant material

The Columbia (Col-0) ecotype of *Arabidopsis thaliana* (L.) Heynh was used as the wild type. Mutants of *mca1* and *mca2* are as described previously (Nakagawa et al. 2007; Yamanaka et al. 2010). Mutants of *aux1-7* (Pickett et al. 1990), *ein2-1* (Guzmán and Ecker 1990), *ein3-1* (Roman et al. 1995), *coronatine insensitive1-16* (*coi1-16*) (Ellis and Turner 2002), *nonexpresser of PR genes1-1* (*npr1-1*) (Cao et al. 1997), *fer-4* (Escobar-Restrepo et al. 2007), *the1* (Hématy et al. 2007), *bak1* (Li et al. 2002), *bak1-like1* (*bkk1*) (Hecht et al. 2001), *wdl5-2* (Sun et al. 2015), and a transgenic line overexpressing *ERF1* under the control of the cauliflower mosaic virus 35S promoter (Solano et al. 1998) were obtained from the Arabidopsis Biological Resource Center.

Growth condition

Seeds were surface-sterilized by a bleach solution with 0.1% Triton X-100, rinsed three times with water, suspended in 0.1% agar and stored in the dark at 4 °C for 3 days before being sown on 0.8% agar medium containing half-strength

MS (pH 5.7) and 1% sucrose. After germination, seedlings were grown vertically at 22 °C under 16 h light/8 h dark long-day conditions for 1 or 2 days and then transferred to new agar media covered with a 12,000–14,000 MWCO dialysis membrane (Spectra/Por 4, Spectrum Laboratories). The membrane was stirred in water for 10 min, then stirred in a solution containing 2% $NaHCO_3$ and 1 mM EDTA at 60 °C for 30 min and washed three times in autoclaved water for 10 min before use. The transferred seedlings were grown vertically or horizontally for 2 days under long-day conditions (Okamoto et al. 2018).

For treatment with 1-aminocyclopropane-1-carboxylic acid (ACC), seedlings grown vertically under long-day conditions for 2 days were transferred to new agar media containing 100 nM ACC and grown vertically for more 2 days. The root length was measured on digital images using Image J (http://www.rsb.info.nih.gov/ij/).

Time-lapse imaging of the root growth

For observation of root gravitropism, seedlings were grown in advance on vertically placed agar plates for 5 days under long-day conditions, transferred to new plates, and grown vertically for further several hours for acclimation. Gravitropic stimulation was then applied by rotating the plates by 90°. Time-lapse imaging was performed at 10-min intervals for 24 h by D3300 digital SLR camera attached with Micro-NIKKOR 55 mm (Nikon, Tokyo, Japan) under the control of the remote timer switch N3 (Etsumi, Tokyo, Japan). The root tip angle was defined as the angle formed between the root tip axis and vertical direction indicated by the root at distal elongation zone, and measured on digital images using Image J.

For observation of root tip bending, seedlings were grown on vertically placed agar plates for 5 days and transferred to a new agar plate. Sterile coverslips of 24 × 60 mm were inserted into the agar plate, perpendicular to both the root growth direction and the surface of the agar medium to form a barrier about 3 mm in front of the growing root tip. Time-lapse imaging was recorded at 15-min intervals for 24 h. The wide angle between the root tip axis and the coverslip was measured at 12 h after the tip reached it using Image J.

Hypoosmotic shock treatment

For the hypoosmotic treatment of seedlings, 5-day-old wild-type and *ein2* seedlings were incubated in 1/2 MS liquid medium containing 1% sucrose and 150 mM mannitol for 20 h, then transferred to the liquid medium without mannitol, and incubated for 0.5 or 2 h.

RNA isolation, cDNA synthesis and qRT-PCR

Total RNA was extracted from whole seedlings using the SDS-phenol method. The total RNA (a 1-µg aliquot) was reverse-transcribed using a PrimeScript II 1st strand cDNA Synthesis Kit (Takara, Kyoto, Japan) with an oligo(dT) primer. qRT-PCR was performed on the Thermal Cycler Dice TP760 (Takara) using KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Wilmington, MA, USA) according to the manufacturer's instruction. *ACTIN8* was used as a control to normalize differences in the amount of total RNA in each sample. Expression of each gene was tested in three biological replicates. The amplified PCR products were verified by melting curve analysis. Intron-spanning primers designed are listed in Supplementary Table S1.

Statistical analyses

Mean values were compared by Student's *t* test between wild-type and mutant plants in Fig. 2 or one-way ANOVA followed by post hoc analysis with Tukey–Kramer multiple tests for the data in other figures. Statistically significant differences are indicated by asterisks for Student's *t* test ($*P < 0.05$) or different letters for one-way ANOVA and Tukey–Kramer test ($P < 0.05$). All statistical analyses were performed using the EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan) (Kanda 2013), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria).

Results

Mutants with altered root growth response to mechanical stress

As shown previously (Okamoto et al. 2008, 2018), wild-type seedlings grown on vertical plates show approximately twofold reduction in the rate of root growth at 2 days after they are transferred to dialysis membrane-covered horizontal plates in comparison with those kept on vertical plates (Fig. 1). Under these experimental conditions, the *aux1* mutant, which is defective in an auxin influx carrier and shows agravitropic root growth, and the ethylene-insensitive mutant *ein2* show no obvious reduction in the root growth rate (Fig. 1; Okamoto et al. 2018). A mutant of *EIN3*, which is a key transcription factor that acts downstream of EIN2 in the ethylene response (Guo and Ecker 2003), also exhibited no growth reduction (Fig. 1). Since exogenous supply of SA attenuates the root growth reduction after mechanical stimulation (Okamoto and Takahashi 2019), the response of *npr1*, which is defective in the SA signaling pathway

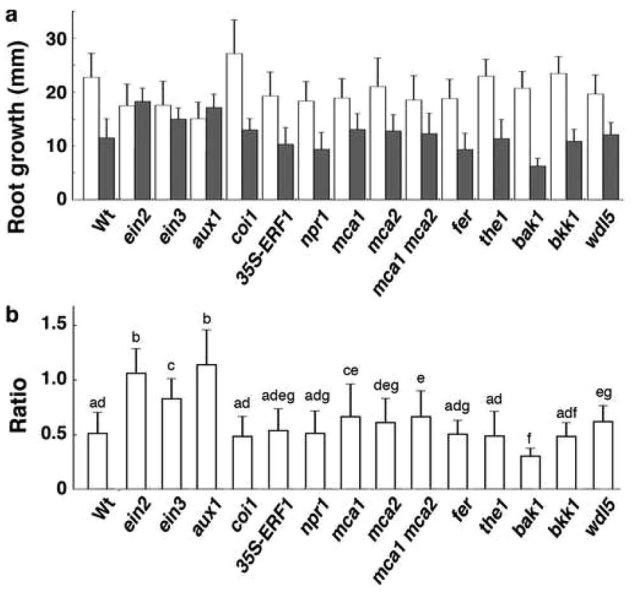

Fig. 1 Effect of different mutations on root growth reduction under mechanical stress conditions. **a** Net root growth for 2 days after transfer of 2-day-old seedlings grown on vertical plates to vertical (white bars) or horizontal (gray bars) plates covered by a dialysis membrane. Error bars correspond to ± SD ($n = 40$); **b** ratio of horizontal to vertical growth in **a**. Different letters indicate statistically significant differences according to one-way ANOVA with Tukey–Kramer multiple comparison test ($P < 0.05$)

(Cao et al. 1997), was examined but no significant alteration from the wild type was observed (Fig. 1). The response of the JA receptor mutant *coi1* (Ellis and Turner 2002) and a transgenic plant line overexpressing *ERF1* under the 35S promoter line (Solano et al. 1998) was also examined. These plant roots showed normal growth response (Fig. 1). These results suggest that the root growth response to mechanical stress is uncoupled from SA, JA, and ERF1-mediated signaling pathways.

We next examined mutants of mechanosensitive Ca^{2+} channels, *mca1* and *mca2* (Nakagawa et al. 2007). While *mca2* roots showed normal growth response, *mca1* and *mca1 mca2* double mutant roots showed a slight but significant decline in the growth reduction compared with that of wild-type roots (Fig. 1), suggesting a role of MCA1 in this response.

To explore the involvement of receptor signaling in the root growth response to mechanical stress, we further examined mutants of *FER* and *THE1*, both of which are known to have a role in cell wall sensing**,** but they showed normal root growth reduction (Fig. 1). On the other hand, the *bak1* mutant showed a significant enhancement of the root growth reduction while a mutant of *BKK1/SERK4*, a paralog with a redundant function to *BAK1* (He et al 2007) showed a normal growth response (Fig. 1).

On the basis of growing evidence that a microtubule-stabilizing protein WDL5 mediates EIN3 signaling (Sun et al. 2015; Dou et al. 2018), we also examined the response in the *wdl5* mutant. The growth reduction after mechanical stimulation was alleviated in *wdl5* roots (Fig. 1).

Kinetics of root gravitropism and bending of the root tip

In our system, gravitropic response is the first and essential step for the root tip to perceive mechanical stress from impenetrable membrane-covered agar. We, therefore, observed kinetics of root gravitropism to examine whether it is affected in the mutants or not. When the plates were rotated from vertical to horizontal position within the vertical plane, it took about 6–8 h for wild-type roots to be redirected downward (Fig. 2). The agravitropic *aux1* mutant roots exhibited a slight reduction in the angle, while the roots of *ein2*, *ein3*, *mca1 mca2*, *bak1*, and *wdl5*, showed

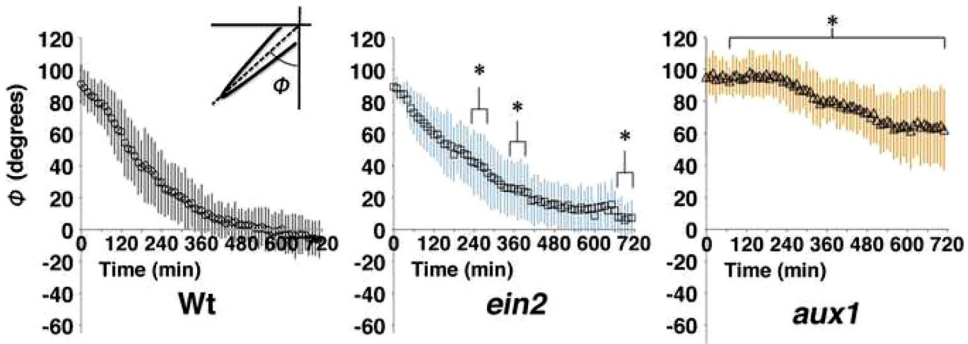

Fig. 2 Kinetics of root gravitropism. Five-day-old wild-type (Wt), *ein2*, *ein3*, *aux1*, *mca1 mca2*, *bak1*, and *wdl5* seedlings were grown on vertical plates and the plates were rotated for the primary root to be oriented horizontally. Time course change in the angle of the primary root from vertical axis was measured by time-lapse photography. Error bars correspond to ± SD ($n = 10$). Asterisks indicate statistically significant differences compared to the wild type at the same time point by Student's *t* test (*$P < 0.05$)

almost the same kinetics as that observed in the wild type (Figs. 2 and S1).

When mechanically impeded, the root tip growing downward to gravity is bent at a certain angle and keeps growing along the obstacle (Massa and Gilroy 2003; Shih et al. 2014). We confirmed that, after being blocked by coverslips, vertically growing Arabidopsis roots had their tips bent and began to slide on the coverslip surface (Movie S1). It took about 8 h from contact to onset of the sliding growth. We then measured the bending angle of the root tip in each mutant after the blocking of the root growth by coverslips. The bending angle was about 140° in the wild type. While the angle was about 160° in *aux1*, it was not significantly affected in other mutants with altered growth response including *ein2*, *ein3*, *mca1 mca2*, *bak1*, and *wdl5* (Fig. 3). Thus, these mutants except *aux1* apparently encounter the same mechanical loads in the root tip under our experimental conditions.

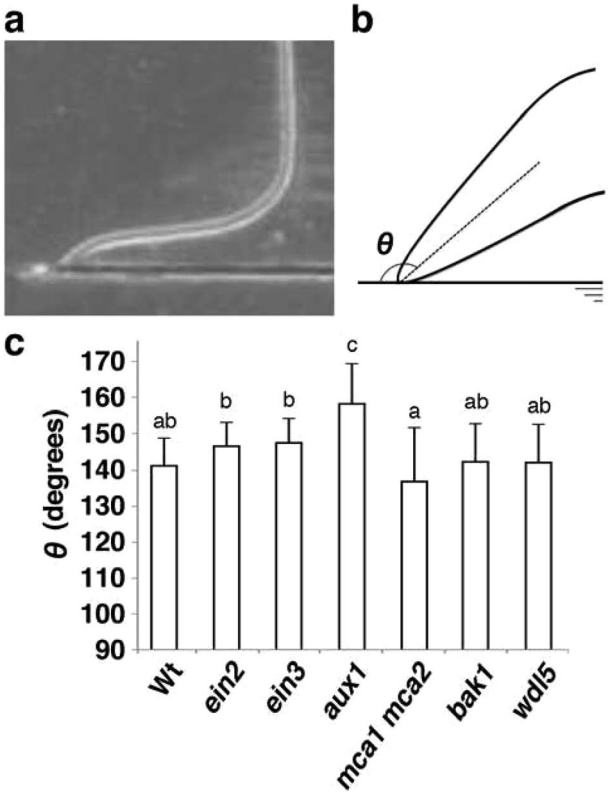

Fig. 3 Effect of mechanical impedance on the bending of the root tip. **a** An image of the root of the seedlings grown for 5 days on vertical plates and then mechanically blocked by coverslips. **b** Schematic illustration of the root tip under mechanical impedance. **c** The root tip angle from horizontal shown as θ in **b**. The angle was measured at 6–12 h after blocking of the root growth by coverslips and averaged. Error bars correspond to ± SD ($n = 10$). Different letters indicate statistically significant differences according to one-way ANOVA with Tukey–Kramer multiple comparison test ($P < 0.05$)

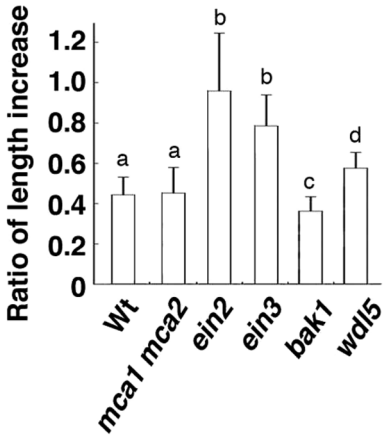

Fig. 4 Effect of ACC on the root growth. Five-day-old seedlings grown on vertical plates were transferred to plates without or with 100 nM ACC for more 2 days. The bars indicate the ratio of length increase of the root grown for 2 days with ACC relative to that without ACC. Error bars correspond to ± SD ($n = 30$). Different letters indicate statistically significant differences according to one-way ANOVA with Tukey–Kramer multiple comparison test ($P < 0.05$)

ACC activates mechanical stress signaling

We next examined whether the altered root growth response to mechanical stress can be reproduced by exogenous treatment of each mutant root with an ethylene precursor ACC or not. When wild-type seedlings grown in vertical plates were transferred to those containing 100 nM ACC, approximately 0.45-fold reduction in the growth was observed in wild-type roots at 2 days after transfer (Fig. 4). Both *ein2* and *ein3* roots showed no significant reduction while *mca1 mca2* roots showed almost the same reduction as that of wild-type roots (Fig. 4), confirming that the Ca^{2+} influx occurs upstream of the action of ACC. On the other hand, the growth reduction was enhanced in *bak1* and attenuated in *wdl5* (Fig. 4). These results suggest that, in place of the mechanical stress, exogenous ACC can activate ethylene signaling in the root growth response.

Expression of mechano-responsive genes in *ein2* and *bak1*

Finally, we performed RT-PCR experiments to know whether expressions of mechanical stress-related genes are affected by the *bak1* mutation or not. According to previous studies showing that hypoosmotic stress can mimic mechanical stimuli (Shih et al. 2014; Tsugama et al. 2016), hypoosmotic treatment of whole seedlings was used as a substitute for mechanical stress treatment of the root tip to enable the cells to respond rapidly and synchronically. Three touch-induced genes, *TCH4* encoding an extracellular xyloglucan

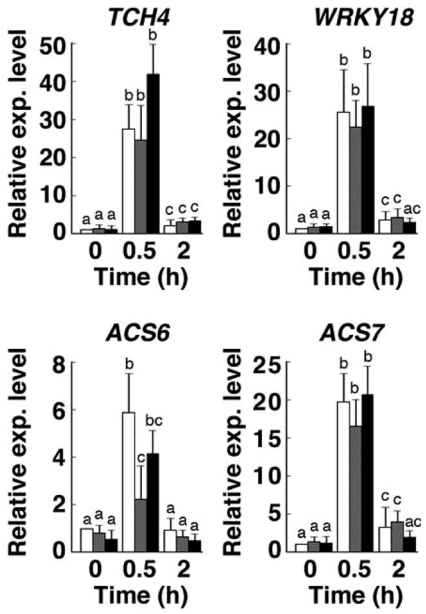

Fig. 5 Expression analysis of *TCH4*, *WRKY18*, *ACS6*, and *ACS7* by qRT-PCR. RNA was prepared from wild-type (white bars), *ein2* (gray bars), and *bak1* (black bars) seedlings treated with hypoosmotic shock for 0, 0.5, and 2 h. *ACTIN8* was used as the internal control. Error bars correspond to ± SD ($n = 3$). Different letters indicate significant differences between groups by one-way ANOVA with Tukey–Kramer multiple comparison test ($P < 0.05$)

endotransglycosylase (Lee et al. 2005), *WRKY18* encoding a transcription factor (Shih et al. 2014), *ACS6* encoding an ACC synthase (Arteca and Arteca 1999), and an osmotic stress-induced *ACS7* (Wang et al. 2005) were examined. Expressions of these genes were transiently and simultaneously induced in 0.5 h after the hypoosmotic treatment of wild-type seedlings and no significant differences in the expression levels were detected in *bak1* (Fig. 5). Similar expression patterns were confirmed in *ein2*, except that the induced level of *ACS6* was slightly lower in *ein2*.

Discussion

To find new components involved in the mechanical signal perception and transmission in Arabidopsis roots, we examined here the root growth response to mechanical impedance of the mutants of hormone signaling, calcium channels, and receptor-like kinases with possible implications using a dialysis membrane-covered agar plate. Since the *aux1* root is insensitive to gravity and the slight reduction in the angle shown in Fig. 2 might be attributed to the dead weight, it may perceive little or no mechanical stress from horizontal agar plates. *ein2* and *ein3* roots have normal gravitropism and no growth response in these mutant roots confirms a pivotal role of ethylene signal transduction in the mechanical response. The root growth reduction after the mechanical stimulation was attenuated in *mca1* and *wdl5* while it was intensified in *bak1*. Since all these mutants examined here except *aux1* showed a normal response to gravity and a normal bending structure of the root tip whose bending angle after contacting coverslips was about 140° in agreement with a previous study (Massa and Gilroy 2003), the altered mechanical stress responses are attributable to defects in the signaling cascades. The result that the growth reduction was not completely reversed in *mca1 mca2* suggests the involvement of additional players to MCA1 and MCA2 in gating Ca^{2+} entry under mechanical stimulation besides the possibility that pure mechanical aspects such as turgor pressure might be affected in the mutant. Potential candidates include members of cyclic nucleotide-gated cation channels (CNGCs), some of which have been shown to be Ca^{2+} permeable, and those of the reduced hyperosmolality-induced [Ca^{2+}] increase (OSCA) family (Dodd et al. 2010; Swarbreck et al. 2013; Hamant and Haswell 2017). OSCA1 was identified as a channel responsible for osmotic stress-evoked Ca^{2+} influx in Arabidopsis (Yuan et al. 2014). Further studies with multiple mutants of these channel genes will help to identify other players of the Ca^{2+} entry in the root response to mechanical stress.

Our finding that the responses to mechanical stress and ACC were attenuated in *wdl5* suggests WDL5 as a likely transducer linking ethylene signaling and root growth. The phenotype is reminiscent of that observed in *wdl5* hypocotyls treated with ACC under darkness (Sun et al. 2015). Ethylene-induced cortical microtubule reorientation and bundling are partially suppressed in *wdl5* hypocotyls (Ma et al. 2016). Probably, the same might occur in the response to mechanical stress in *wdl5* roots. There are a number of plant-specific microtubule-associated proteins involved in cell elongation (Hamada 2014) including the WDL family (Perrin et al. 2007; Lian et al. 2017). These proteins might also play a fundamental role in microtubule dynamics during the root growth response.

Enhancement of the growth reduction in *bak1* suggests the involvement of receptor-mediated signal transduction in this response. Importantly, supplementation of ACC also enhanced the root growth reduction in the absence of mechanical stress in *bak1* while gene expressions of ethylene synthesizing enzymes, ACS6 and ACS7, were normally induced by hypoosmotic treatment of *bak1* seedlings. These results suggest that BAK1 acts downstream of ethylene signaling. BAK1 is a co-receptor and plays versatile roles in the perception of various extracellular ligands including brassinosteroids (Russinova et al. 2004), bacterial flagellin (Chinchilla et al. 2007), and phytosulfokine (PSK) (Ladwig et al. 2015). In the response to PSK, BAK1 forms a functional complex with a PSK receptor PSKR1, CNGC17, and plasma membrane-localized H^+-ATPases AHA1 and AHA2 to link

proton extrusion to cation uptake, resulting in the promotion of cell growth (Ladwig et al. 2015). Another study reveals that BAK1 phosphorylates CNGC20 and results in its low abundance and the containment of Ca^{2+}-induced cell death (Yu et al, 2019). Thus, BAK1 may have opposite functions in regulating different CNGC interactions. Our results suggest the possibility that BAK1 and probably its interacting proteins are involved in the recovery from the root growth cessation or the desensitization of ethylene signaling after ethylene production. Among RLKs, members of the CrRLK1L subfamily including FER and THE1 are known as potential cell wall sensors (Franck et al. 2018). However, mutants of *fer* and *the1* showed no altered root growth response in our system. According to a previous study (Shih et al 2014), *fer* mutants exhibit defective growth responses to mechanical perturbation and also an altered bending angle of the root tip on coverslips. It is thus possible that the mechanical stress perceived at the root tip in our system using dialysis membrane is too weak to cause the impairment of cell wall integrity that is detectable by such cell wall sensors as FER or THE1.

In conclusion, altered root growth responses observed in *bak1* and *wdl5* suggest that additional components can be further identified by research of the mutants of RLKs and microtubule-related proteins or by screening for new mutants in our experimental system.

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Author contribution statement TO and ST performed the experiments. TO, HM and TT designed the research, analyzed the data and carried out statistical analyses. TO, HI and TT wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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