Mild salinity stimulates a stress-induced morphogenic response in \textit{Arabidopsis thaliana} roots

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Abstract

Plant roots exhibit remarkable developmental plasticity in response to local soil conditions. It is shown here that mild salt stress stimulates a stress-induced morphogenic response (SIMR) in \textit{Arabidopsis thaliana} roots characteristic of several other abiotic stresses: the proliferation of lateral roots (LRs) with a concomitant reduction in LR and primary root length. The LR proliferation component of the salt SIMR is dramatically enhanced by the transfer of seedlings from a low to a high NO$_3$ medium, thereby compensating for the decreased LR length and maintaining overall LR surface area. Increased LR proliferation is specific to salt stress (osmotic stress alone has no stimulatory effect) and is due to the progression of more LR primordia from the pre-emergence to the emergence stage, in salt-stressed plants. In salt-stressed seedlings, greater numbers of LR primordia exhibit expression of a reporter gene driven by the auxin-sensitive \textit{DR5} promoter than in unstressed seedlings. Moreover, in the auxin transporter mutant \textit{aux1-7}, the LR proliferation component of the salt SIMR is completely abrogated. The results suggest that salt stress promotes auxin accumulation in developing primordia thereby preventing their developmental arrest at the pre-emergence stage. Examination of ABA and ethylene mutants revealed that ABA synthesis and a factor involved in the ethylene signalling network also regulate the LR proliferation component of the salt SIMR.

Key words: ABA, \textit{Arabidopsis thaliana}, auxin, lateral root, salt stress, SIMR.

Introduction

A major feature of plant development is that it is continuously modified by interactions with the environment thereby enabling plants to cope with the constraints of their sessile lifestyle. A quintessential example is the developmental plasticity of the three-dimensional deployment of the root (the root architecture) in response to the soil environment. This plasticity is critical for a plant’s ability to forage the soil for nutrients and water and contributes to a plant’s competitive fitness (Grime \textit{et al.}, 1986; Lynch, 1995). Root architecture is determined by the pattern of root branching (lateral root formation) and by the rate and direction of growth of individual roots (Malamy, 2005). The formation of lateral roots is a reiterative process. Lateral roots (LRs) form along the length of the primary root and these first order LRs give rise to second order LRs which themselves can form third order LRs etc. In \textit{Arabidopsis thaliana}, LRs initiate from a subset of root pericycle cells adjacent to the two xylem-poles (Dubrovsky \textit{et al.}, 2000, 2001; Casimiro \textit{et al.}, 2001). These ‘founder’ cells undergo successive cell divisions to generate a dome-shaped structure of cells, the LR primordium (LRP). After establishment of a new meristem layer, the LRP expands and emerges through the epidermis of the parent root (Bhalerao \textit{et al.}, 2002).

Several studies have shown how differences in the composition of the growth medium can alter root architecture (reviewed in Malamy, 2005). A good example is the proliferation of LRs into resource-rich growth media zones such as those enriched in NO$_3$ or phosphate (Drew \textit{et al.}, 1973; Drew and Saker, 1975; Zhang and Forde, 1998; Zhang \textit{et al.}, 1999; Malamy and Ryan, 2001; Linkohr \textit{et al.},...
202). On the other hand, less is understood regarding how plant root architecture is modified in response to stressful conditions, even though this response may be vital for the plant’s ability to compete for soil resources. A recent survey of plant responses to heavy metal stress, phosphorus deficiency, UV or mechanical stress indicated a common morphogenic response characterized by the inhibition of primary root or shoot elongation and enhanced production of LRs or axillary branching (Potters et al., 2007, 2009). The authors suggested the existence of a co-ordinated, generic ‘stress-induced morphogenic response’ (SIMR), which is stimulated by mild chronic stress and comprises (i) inhibition of elongation; (ii) localized stimulation of cell division; (iii) alterations in cell differentiation status. The SIMR is thus typified by growth redistribution rather than by the cessation of growth.

The response of roots to drought or salinity stress is less clear. Several reports have described the reduction in root elongation caused by the application of osmotic or salinity stress in cotton, maize, and bean (Kurth et al., 1986; Itoh et al., 1987; Sharp et al., 1988; Eshel and Waisel, 1996). In cotton and in seedlings of three boreal conifers, a reduction in LR number is also observed (Eshel and Waisel, 1996; Croser et al., 2001). In most studies using Arabidopsis, osmotic or salt stress severely represses the formation of LRs (Burssens et al., 2000; van der Weele et al., 2000; Kim et al., 2004; West et al., 2004; Deak and Malamy, 2005; Xiong et al., 2006). Osmotic stress appears to inhibit LR formation after LR initiation and affects the formation of autonomous LRs from LRP s (Deak and Malamy, 2005). However, during their studies of AtNAC2, a transcription factor involved in LR proliferation, the dose response of LR proliferation, the dose response of AtNAC2 root architecture, it was decided to investigate this question in more detail. In the present study, it is reported that mild salt stress causes a drastic reduction in primary root elongation and LR elongation but an increase in LR number, constituting a typical SIMR. Enriching the medium with NO$_3^-$ dramatically enhances the effects of salt stress on LR number such that the overall LR surface area for absorption is maintained. The SIMR is specifically caused by salt stress but not by osmotic stress alone. Stimulation of LR number by salt stress is due to the progression of more LRPs from the pre-emergence to the emergence stage of LR formation. Evidence is also presented suggesting that the LR proliferation component of the SIMR is mediated by auxin accumulation in developing LRPs and by the stress hormone ABA.

**Materials and methods**

*Plant material and growth conditions*

All wild-type and mutant Arabidopsis thaliana lines were grown in a Columbia background. Seeds were surface-sterilized in NaClO (3% w/v) for 5 min, rinsed five times with sterile water, and sown on plates containing seedling nutrient medium (Wilson et al., 1990) including 1 mM KNO$_3$. Seeds were stratified at 4 °C for 4 d and then placed vertically in a growth room at 22 °C, with a 16/8 h light/dark photoperiod. When primary root length was approximately 2 cm (4–5 d), seedlings were transferred to the top segment of a vertical glass plate divided into three segments by glass dividers with each segment containing nutrient media supplemented with 1 mM KNO$_3$, plus/minus 50 mM NaCl or 100 mM mannitol (100 mM mannitol has an approximate equal osmolarity to 50 mM NaCl). Enough medium was added so that roots were able to grow from one segment to another and the top edge of the agar in the top segment was cut away before the transfer of seedlings to ensure that only roots, not shoots, were in contact with the medium (MacGregor et al., 2008). For low to high NO$_3^-$ treatments, seeds were initially sown on basal nutrient medium containing 10 μM KNO$_3$ instead of 1 mM KNO$_3$.

**Analysis of root growth**

For visualization, roots were stained with Brilliant Cresyl Blue as described by Gruntman and Novoplansky (2004). Roots were photographed with a C-5050 ZOOM digital camera (Olympus, Center Valley, PA). For LRP analysis, seedlings were harvested 8 d after transfer to segmented plates, stained with 2% (w/v) acetoarmine (Sigma-Aldrich, St Louis, MO) for 30 min at 78 °C and then cleared and mounted according to Malamy and Benfey (1997). For histochemical staining of GUS activity, seedlings were collected in staining buffer (0.1 M TRIS pH 7.5, 2.9 mg ml$^{-1}$ NaCl, 0.66 mg ml$^{-1}$ K$_2$Fe(CN)$_6$, and 20% methanol) and stained for 5 d as described by Malamy and Benfey (1997). Roots sections were photographed with a digital camera DXM1200F (Nikon Instruments, Badhoevedorp, Netherlands) mounted on a Nikon Eclipse TE2000-U microscope. For experiments carried out for 21 d, data were statistically analysed using the Fisher’s protected LSD test. However, for experiments carried out for 8 d or 12 d, and where the total number of LRs produced was much smaller, the more stringent Tukey’s post-hoc test was performed.

**Results**

*Mild salt stress inhibits primary and lateral root elongation and stimulates LR proliferation*

To examine how salt stress affects Arabidopsis LR proliferation, the dose response of Arabidopsis seedlings to increasing salt concentration was tested. Seedlings were germinated and grown on vertical nutrient agar plates until the root was ~2 cm long (4–5 d) and then transferred to fresh plates with increasing levels of NaCl. At the time of transfer no LRs were visible. Figure 1 shows that increasing NaCl levels up to 50 mM stimulated the production of LRs, where seedlings exhibited virtually twice the number of first order LRs (emerged LRs) after 21 d than the control (0 mM NaCl) and also produced a few second order LRs (data not shown). However, the transfer of seedlings to 100 mM salt stress caused almost complete inhibition of LR development. These results suggest an acclimation response of LR development to mild levels of salt stress.

To investigate the stimulatory effect of mild salt stress on LR development further, 4–5 d-old seedlings were transferred to vertical segmented nutrient agar plates with or without 50 mM NaCl (the NaCl concentration that had displayed the greatest stimulatory effect) in all the segments. It was observed that first order LR number increased significantly in the top and middle segments of the plate compared with unstressed plants (Fig. 2A). Salt stress...
caused an even greater stimulation of second order LR proliferation where stressed seedlings produced 8-fold more LRs than unstressed seedlings (Fig. 2B). Furthermore, only stressed plants developed third order LRs (Fig. 2C). By contrast with its stimulatory effect on LR number, salt stress led to a reduction in average LR length (Fig. 2D) with stressed LRs exhibiting approximately half the length of unstressed LRs. Moreover, although salt stress led to an increase in LR number, an evaluation of total LR length (a function of both total LR number and average LR length) suggested that the salt-mediated increase in LR number could not fully compensate for the reduction in the average LR length (Fig. 2E). Primary root elongation was severely reduced in stressed seedlings, which displayed a 4-fold reduction in primary root length compared with unstressed seedlings (Fig. 2F). Indeed, only unstressed primary roots reached the bottom segment of the plate, hence the absence of any first order stressed LRs in the bottom segment (Fig. 2A). Overall, our results suggest that mild salt stress stimulates an SIMR phenotype in contrast to severe salt stress where LR proliferation is inhibited.

Stimulation of LR proliferation by salt stress is enhanced upon transfer from low to high NO₃

The stimulatory effect of nitrate-enriched media on LR proliferation has been well documented (reviewed in Zhang

Fig. 1. Effect of NaCl concentration on Arabidopsis lateral root number. Seedlings grown on 1 mM KNO₃ medium for 4–5 d were transferred to the same medium plus the indicated concentrations of NaCl for 21 d. Data are mean emerged LRs ± SE (n=30). Bars with different letters indicate significant difference at \( P \leq 0.05 \) (Fisher’s protected LSD test). The data are representative of similar results in three independent experiments.

Fig. 2. Salt stress causes an SIMR phenotype. (A–F) Seedlings grown on 1 mM KNO₃ medium for 4–5 d were transferred to the same medium ±50 mM NaCl for 21 d. Data are mean ± SE (n=20). * Significant difference \( (P \leq 0.05) \) between unstressed and stressed roots (Fisher’s protected LSD test). The data are representative of similar results in three independent experiments. Top, Middle, Bottom: the different segments of the segmented agar plate.
et al., 2007). In Arabidopsis, the transfer from NO₃⁻-poor to NO₃⁻-rich medium predominantly stimulates LR elongation (Zhang et al., 1999; Linkohr et al., 2002). It was therefore tested whether transfer from low NO₃⁻ to NO₃⁻-rich medium would have a stimulatory effect on the LR component of the salt SIMR, by transferring seedlings grown on 10 μM KNO₃ to plates containing 1 mM KNO₃ with and without NaCl uniformly present across all plate segments. The enrichment of the NO₃⁻ supply led to a dramatic enhancement of the salt-induced increase in LR number. While first order LRs were induced to a similar extent as with salt alone (compare Figs. 2A and 3A) the mean number of second and third order LRs (16 and 8, respectively) exhibited a striking enhancement compared to salt stress alone (6.5 and 1.5, respectively) (compare Figs. 2B and 3B; 2C and 3C). On the other hand, when combined with salt treatment, the enrichment of the NO₃⁻ supply had no stimulatory effect on LR elongation (Fig. 3D); mean LR length was reduced to a similar degree as with salt stress alone (compare Figs. 2D and 3D). Strikingly, evaluation of total LR length showed that NO₃⁻ enrichment of the salt-stress-induced increase in LR number fully compensated for the decreased average LR length (Fig. 3E). Primary root elongation was again severely inhibited in salt-stressed roots (Fig. 3F).

Salt stress comprises two stress components, namely osmotic stress and ionic stress. To determine whether the salt SIMR is specific to salt stress and not to osmotic stress alone, the effect of the latter on root architecture was tested. When seedlings were transferred from 10 μM KNO₃ to 1 mM KNO₃ supplemented with 100 mM mannitol, primary root elongation was only minimally affected by osmotic stress (compare Figs. 3F and 4E). Furthermore, instead of a stimulation of LR proliferation, a reduction in first order LR number was observed in stressed roots compared with unstressed roots (Fig. 4A). Moreover, second order LRs only appeared in unstressed roots (Fig. 4B) while no third order LRs were observed in either unstressed or stressed roots. The average LR length was reduced by osmotic stress in a similar manner to salt stress (Fig. 4C) and this reduction in average LR length combined with the decreased LR number led to a large fall in total LR length (Fig. 4D). Similar results were obtained when seedlings were transferred to mannitol but without the stimulatory effect of NO₃⁻ enrichment (transfer of seedlings from 1 mM KNO₃ to 1 mM KNO₃ with or without mannitol [data not shown]). These findings show that osmotic stress alone or in combination with NO₃⁻ enrichment is not sufficient to stimulate a characteristic SIMR.

Fig. 3. The LR proliferation component of the salt SIMR is enhanced by transfer from low to high NO₃⁻. (A–F) Seedlings grown on 10 μM KNO₃ medium for 4–5 d were transferred to 1 mM KNO₃ medium ±50 mM NaCl for 21 d. Data are mean ±SE (n=20). * Significant difference (P < 0.05) between unstressed and stressed roots (Fisher’s protected LSD test). The data are representative of similar results in three independent experiments. Top, Middle, Bottom: the different segments of the segmented agar plate.
Greater numbers of LRPs develop into LRs during the $\text{NO}_3^-/C_0$-enhanced salt SIMR

It was next investigated whether development of LR primordia (LRPs) is affected during the $\text{NO}_3^-/C_0$-enhanced salt SIMR. Seedlings were again grown on 10 $\mu$M $\text{KNO}_3$ and transferred to plates containing 1 mM $\text{KNO}_3$ with and without NaCl uniformly present across all plate segments. Visualization of LRPs was performed by staining with acetocarmine (Enstone et al., 2001) and the development of each primordium assessed by combining the detailed stages of LRP development assigned by Malamy and Benfey (1997) into four broad phases: (INT) initiation, (CP) cell proliferation, (PE) pre-emergence, and (E) emergence (Fig. 5A). Almost 60% of first order LRPs reached the E stage in stressed seedlings whereas the greatest proportion (42%) of LRPs in unstressed plants was at the PE stage of development (Fig. 5B). Although similar numbers of INT stage LRPs in unstressed and stressed seedlings were observed, no CP stage LRPs in stressed seedlings were detected compared to 21% CP stage LRPs from unstressed seedlings. Similar results were observed with second order LRPs although, as expected, a greater percentage of LRPs from both unstressed and stressed seedlings were in the earlier stages of development than first order LRPs (Fig. 5C). Nevertheless, the highest proportion of second order LRPs in stressed plants was at the E stage of development whereas the greatest percentage of LRPs in unstressed plants was at the PE stage. These results suggest that while the proportion of LRPs being initiated in both unstressed and stressed plants is similar, the overall increase in LR number in stressed roots (Fig. 5D) is either due to the arrest of a proportion of LRPs in unstressed plants at the pre-emergence stage of development or, alternatively, LRPs in stressed roots develop at a faster rate.

$\text{NO}_3^-/C_0$-enhanced, salt stress stimulation of LR proliferation is mediated by auxin

One of the major generic characteristics of root SIMR phenotypes is the increased proliferation of LRs (Potters et al., 2007). Auxin is a key regulator of LR development. It promotes LRP initiation by the local activation of root pericycle cells and also plays a role in LR emergence (Reed et al., 1998; Casimiro et al., 2001; Bhalerao et al., 2002; Marchant et al., 2002; De Smet et al., 2007; Okushima et al., 2007; Dubrovsky et al., 2008). It was therefore investigated whether the LR proliferation component of the
NO$_3^-$-enhanced salt SIMR is mediated by auxin, by testing if salt stress can still induce LR proliferation in (i) an auxin synthesis mutant, nit1-3 (Normanly et al., 1997) [NIT1 is one of three nitrilases that catalyse a biosynthetic step in one of several IAA biosynthesis pathways (Woodward and Bartels, 2005)]; (ii) auxin transport mutants, axr4-2, aux1-7 (Picket et al., 1990; Dharmasiri et al., 2006); (iii) auxin signalling mutants, axr1-3, tir1-1, eta3/tir1-1 (the eta3 background enhances the tir-1-1 phenotype) (Lincoln et al., 1990; Ruegger et al., 1998; Gray et al., 2003; Dharmasiri et al., 2005; Kepinski et al., 2005). All auxin mutants exhibited decreases in either, or both, unstressed and stressed LR number compared to wild-type (Fig. 6A). However, while the stimulation of LR proliferation under salt stress was observed in the axr1-3, axr4-2, nit1-3, tir1-1, and eta3/tir1-1 mutants, it was completely blocked in the aux1-7 mutant, suggesting that auxin transport, at least via AUX1, is essential for the LR proliferation component of the salt SIMR.

A clear trend showing a stress-induced decrease in average LR length could be observed in wild-type, axr1-3, axr4-2, nit1-3, and aux1-7 roots, although this decrease was not statistically significant except for the nit1-3 and aux1-7 mutants (Fig. 6B). No significant salt-mediated reduction in average LR length could be seen in the tir1-1 mutant, but this was due to a large decrease in unstressed LR elongation with little effect on stressed LR length. This phenotype was enhanced in the eta3/tir1-1 mutant. All mutants exhibited the wild-type inhibition of primary root elongation by salt stress (Fig. 6C). Overall, the results suggest that auxin does not play a role in the root elongation component of the salt SIMR but is important for LR elongation under unstressed conditions.

Our results indicate that at least auxin transport is essential for the salt-mediated stimulation of LR proliferation. AUX1 is an influx carrier that imports auxin into developing LRP to regulate both LRP initiation and emergence (Marchant et al., 1999, 2002; Swarup et al., 2001; Yang et al., 2006). To test whether the LR proliferation component of the NO$_3^-$-enhanced salt SIMR is associated with increased auxin accumulation, expression of the GUS reporter gene, driven by the auxin-sensitive DR5 promoter, was examined in developing LRP. DR5 is an established indirect marker for auxin accumulation and is expressed from the earliest stages of LRP development (Ulmasov et al., 1997; Benkova et al., 2003; Dubrovsky et al., 2008). It was possible to detect LRP developmental stages INT to E by histochemical staining for GUS activity (Fig. 6D). Remarkably, only 20% of LRP in unstressed seedlings expressed GUS staining while 70% of LRP in stressed seedlings were stained (Fig. 6E). Moreover, twice the number of LRP per root system was observed in unstressed seedlings compared to stressed seedlings, whereas twice as many LRs had developed in stressed seedlings (Fig. 6F and inset in E). Taken together, these results suggest that the development of a greater number of LRP is arrested in the absence of salt stress; the corollary being that salt stress stimulates the development of a larger proportion
of LRPs and that this stimulation is associated with increased auxin accumulation in the LRPs. This finding correlates well with our observation that, while a similar proportion of LRPs in unstressed and stressed seedlings are initiated, a greater percentage of LRPs in roots of stressed seedlings reach developmental stage E than in unstressed seedlings where a proportion of LRPs appear to be arrested at the CP or PE stages (Fig. 5B, C).

Fig. 6. Salt stress affects auxin distribution in LRPs. (A–C) Total LR number, average LR length, and primary root length, respectively, of WT and auxin mutants transferred from 10 μM KNO₃ to 1 mM KNO₃ medium ±50 mM NaCl for 8 d. Data are mean ±SE (n=20). * Significant difference (P<0.01) between unstressed and stressed roots (Tukey’s post-hoc test). (D) LRP development visualized by DR5:GUS staining. Growth conditions were as described (Benkova et al., 2003). (E) Frequency of stained LRP. (F) LRP number per root system. Inset in (E), Total LR number. For (E), (F) and inset, data are mean ±SE (n=30). * Significant difference (P<0.05) between unstressed and stressed roots (Fisher’s protected LSD test). The data are representative of similar results in three independent experiments.
ABA and an ethylene signalling factor regulate NO₃−/Co₂−-enhanced, salt stress stimulation of LR proliferation

In contrast to auxin, ABA exerts an inhibitory effect on LR development at the post-emergence stage (De Smet et al., 2003, 2006). Evidence suggests that ABA action on root development may require, at least in part, a functioning ethylene signalling network and that ABA inhibition of root growth is attenuated in ethylene signalling mutants such as ein2 and etr1 (Beaudoin et al., 2000; Ghassemian et al., 2000; Cheng et al., 2002). It was therefore investigated whether ABA or ethylene signalling play a role in the NO₃−-enhanced salt SIMR by testing whether salt stress leads to a different phenotype from the wild type in the following ABA and ethylene mutants: (i) ABA synthesis mutants, aba2-1, aba3-1 (Leon-Kloosterziel et al., 1996; Schwartz et al., 1997); (ii) the ABA signalling mutant, abi4-1 (Finkelstein, 1994; Soderman et al., 2000); (iii) ethylene signalling mutants, ein2-1, etr1-3 (Guzman and Ecker, 1990; Chang et al., 1993).

The aba2-1 mutant produced a greater number of first order LRs than the wild type in both unstressed and stressed seedlings (Fig. 7A) consistent with the inhibitory role of ABA in LR development. However, NO₃−-enhanced salt stress stimulation of first order LR proliferation was completely abrogated in this mutant. The slightly weaker aba3-1 mutant (Leon-Kloosterziel et al., 1996) also displayed no significant difference in first order LR number between unstressed and stressed seedlings. The ein2-1 mutant exhibited a similar phenotype to the aba2-1 mutant with greater numbers of first order LRs in unstressed and stressed seedlings but no stimulation of first order LR proliferation in stressed seedlings. By contrast, salt stress stimulated first order LR proliferation in the abi4-1 and etr1-3 mutants. These results suggest that ABA synthesis is required for the first order LR proliferation component of the salt SIMR, that ABI4 is not involved in the signalling network and that at least one factor involved in the ethylene signal network is required.

Figure 7B shows that the response of second order LRs was different from that of first order LRs. Second order LRs were almost only observed in stressed roots in both wild-type and mutant seedlings. Both the aba mutants and the etr1-3 mutant exhibited a similar number of LRs to the wild type, whereas the abi4-1 and ein2-1 mutants displayed greater LR proliferation. These findings suggest that second order LRs are regulated in a different manner to first order LRs.

All the ABA and ethylene signalling mutants exhibited wild-type phenotypes of salt-mediated inhibition of LR and primary root length (Fig. 7C, D) suggesting that the root elongation component of the salt SIMR does not involve ABA or ethylene signalling.

**Discussion**

Several distinct abiotic stresses (heavy metals, UV, phosphorus-deficiency, mechanical stress) lead to a stress-induced morphogenic response (SIMR) which, in roots, consists of the inhibition of root elongation and an increase in LR number (Potters et al., 2007). However, due to conflicting studies in recent years regarding the effect of salt stress on...
Arabidopsis root architecture, it is unclear whether salinity causes a root SIMR. Our data show that mild salt stress leads to a characteristic SIMR: a reduction in Arabidopsis LR and primary root elongation with a concomitant stimulation of LR number and LR branching (Figs. 1, 2). On the other hand, a high level of salt stress leads to a reduction in LR number (Fig. 1) again typical of an SIMR, which is an active process and is therefore inhibited under severe stress (Potters et al., 2007). A closer inspection of the experimental systems used in different salinity studies might account for the contrasting reports of the effect of salt stress on LR development. For instance, one study that showed a salt-mediated reduction in Arabidopsis LR number used 3.5 times the concentration of NaCl used in our system (Burssens et al., 2000). Arabidopsis is a salt-sensitive plant and the reduction in LR number at such high salinity levels is likely to represent salt-induced damage rather than bona-fide acclimation.

The Malamy laboratory has shown the importance of experimental design on the interpretation of LR development data from Arabidopsis grown in culture. This group had shown in a previous paper that osmotic stress leads to a repression of LR formation in culture-grown Arabidopsis (Deak and Malamy, 2005). However, in a recent paper, they demonstrated that uptake of sucrose by aerial tissues in contact with the growth medium stimulated LR development (MacGregor et al., 2008). Moreover, the apparent repression of LR formation by osmotic stress was actually due to an osmotic stress-mediated decrease in the permeability of aerial tissues thereby reducing the uptake of sucrose and causing inhibition of LR development. This finding may also explain the results of a report showing a reduction in Arabidopsis LR number using mild salt stress but where aerial tissues were in contact with sucrose-containing growth medium (Kim et al., 2004). By contrast, our experiments were designed so that the top section of the agar was cut away and only the roots were in contact with the growth media. Our data thus provide strong evidence that the stimulation of LR proliferation under mild salt stress is an acclimation response.

It is not possible at this stage to distinguish whether the ionic or osmotic components of salt stress, or a combination of both, are responsible for the LR proliferation component of the salt SIMR. While it could be shown that osmotic stress (mannitol treatment) was not sufficient to promote an increase in LR number (Fig. 4), further experiments using a salt such as LiCl that imposes ionic toxicity at lower concentrations than NaCl would aid in determining the relative importance of the two components of salt stress (Tester and Davenport, 2003). On the other hand, the osmotic component of salt stress was probably responsible for the inhibition of LR and primary root elongation. This idea is supported by the observation that both salt stress and osmotic stress lead to shorter LRs and primary roots and by the data showing the salt stress stimulation of LR number implying that the inhibition of root elongation was not due to ion toxicity.

Our results show that the LR proliferation component of the salt SIMR is massively enhanced by the transfer of seedlings from low to high NO₃⁻ (Fig. 3). Indeed, the increased LR number fully compensates for the salt stress-mediated reduction in LR length, thereby maintaining the overall LR surface area for absorbing nutrients and water. The stimulatory effect of transfer from low to high NO₃⁻ upon Arabidopsis LR development has been well documented (Zhang and Forde, 1998; Zhang et al., 1999, 2000, 2007; Linkohr et al., 2002). However, the predominant effect is the stimulation of LR elongation. An increase in LR elongation was also noted upon transfer to high NO₃⁻ in the absence of salt, compared to low NO₃⁻, with no effect on LR number (data not shown). Moreover, our data demonstrate that transfer from low to high NO₃⁻ without salt does not lead to LR proliferation. This evidence supports the idea that the signal stimulating an increase in LR number in our experiments is salt stress. Transfer from low to high NO₃⁻ only enhances the salt stress signal. The mechanism by which NO₃⁻ enhances the LR proliferation component of the salt SIMR is unclear. It is possible that the increase in NO₃⁻ supply indirectly promotes LR proliferation by affecting cotyledon and leaf size. However, in transfer experiments where seedlings experienced greater NO₃⁻ supply throughout the experiment leading to bigger seedlings at the time of transfer (e.g. Figs 1, 2: NO₃⁻ supply was kept constant at 1 mM KNO₃ before and after transfer), salt stress stimulation of LR proliferation was less than when smaller seedlings initially grown on low (10 μM) NO₃⁻ were transferred to high NO₃⁻. This suggests that the NO₃⁻-mediated enhancement is due to the plant’s ability to sense changes in the external NO₃⁻ supply upon transfer from low to high NO₃⁻. Such a NO₃⁻ sensing system has been demonstrated in Arabidopsis (Zhang and Forde, 1998; Remans et al., 2006). In this system, the NO₃⁻ ion itself, acts as a signalling molecule and evidence suggests that the nitrate transporter NRT1.1 may act as a key NO₃⁻ sensor regulating expression of ANR1, a MADS box transcription factor thought to transduce the NO₃⁻ signal internally. However, this NO₃⁻ sensing system is associated with NO₃⁻ stimulation of LR elongation. Whether it is also part of the sensing system regulating NO₃⁻ enhancement of the salt stress-mediated increase in LR number requires further investigation.

It was possible to correlate the NO₃⁻-enhanced salt SIMR with the development of more LRPⅵs into LRs than was observed in unstressed roots (Fig. 5). Although speculative, the fact that unstressed roots possess a large proportion of LRPⅵs whose development appears to be arrested suggests that an optimal number of LRs are produced under favourable conditions but that new developing roots are ‘primed’ with an excess of LRPⅵs as a contingency against sub-optimal soil conditions. Seventy per cent of LRPⅵs in stressed seedlings showed DR5:GUS staining compared with 20% of LRPⅵs in unstressed seedlings (Fig. 6E). It has previously been shown that expression from the auxin-responsive DR5 promoter is undetectable in LRPⅵs that fail to develop into LRs (Benkova et al., 2003; De Smet et al.,...
in response to many abiotic stresses and activates the expression of numerous stress-responsive genes (Zeevaart and Creelman, 1988; Seki et al., 2002). Moreover, ABA has a well-documented role in lateral root formation (De Smet et al., 2006). In general, ABA and auxin act antagonistically; whereas auxin acts to promote LR proliferation, ABA has been shown to inhibit LR development by suppression of the post-emergence activation of the LR meristem (De Smet et al., 2003). In our experiments, this inhibitory role of ABA can be observed in the aba2-1 mutant, which exhibits increased first order LR numbers in both unstressed and stressed roots (Fig. 7). However, the abolition of the first order LR proliferation component of the salt SIMR in the aba2-1 and aba3-1 mutants suggests that ABA can also act as a positive signal in LR development. A positive role for ABA in LR development is supported by the demonstration that stimulation of LR elongation upon transfer of seedlings from low to high NO₃⁻ levels is mediated by ABA (Signora et al., 2001). It is thus also conceivable that the enhancement of the LR proliferation component of the salt SIMR by NO₃⁻ could be regulated via ABA.

Our data indicate that ABA does not exert its effect on the first order LR proliferation component of the salt SIMR via the ABA signalling factor, ABI4. However, the stimulation of first order LR proliferation was abrogated in the ein2-1 mutant whose LR phenotype was similar to the aba2-1 mutant. This suggests that the stimulatory effect of ABA on the salt SIMR may involve factors of the ethylene signalling pathway. On the other hand, ethylene may regulate the LR proliferation component of the salt SIMR independently of ABA. For instance, two recent reports suggest that ethylene regulates Arabidopsis root branching via interactions with auxin (Ivanchenko et al., 2008; Negi et al., 2008).

Intriguingly, the response of second order LRs in the wild type, and in ABA and ethylene mutants differed from that of first order LRs. In fact, the higher order LRs showed the greatest NO₃⁻-enhanced, salt stress stimulation of LR number throughout this study suggesting either increased uptake of salt or/and NO₃⁻, or increased sensitivity to the salt-mediated signal, the NO₃ signal or a combination of the two. Little is understood regarding both the intrinsic and extrinsic regulation of higher order LRs and whether, or how, this regulation differs from that of first order LRs. Our results demonstrate the importance of separating the analysis of LRs of different order to begin to dissect the mechanisms that control root branching.

What might be the physiological and ecological significance of the salt SIMR and its enhancement by NO₃⁻-rich growth media? It has been suggested that SIMRs in general are a mechanism for stress evasion and represent a redirection of growth away from localized sources of stress (e.g. heavy metals) or a means to reduce stress exposure (e.g. UV-B penetration) (Potters et al., 2007). This hypothesis could conceivably apply to the salt SIMR as well. On the other hand, the reduction in both LR length and primary root length under salt stress would reduce root surface area for absorption. This would occur at a time...
when a reduction in soil water potential due to increased salt concentration would make it more difficult to maintain water flow into the plant. However, a rise in LR number (the LR proliferation component of the SIMR) would partly compensate for the reduction in LR length by providing greater root surface area for absorption of water and nutrients albeit at a reduced absorption rate. The significance of enhancement of the LR proliferation component of the salt SIMR by NO$_3^-$ may be more complex. It has been shown that an extensive root system is only required for relatively immobile nutrients such as phosphate ions whereas mobile ions such as nitrate can be efficiently taken up by a more restricted root architecture (Robinson, 1996; Fitter et al., 2002). Root proliferation for mobile ions becomes important only when plants are in competition for those ions (Hodge et al., 1999; Robinson et al., 1999). However, Fitter et al. (2002) showed that under extreme conditions of high N and high P, a larger root system might be important for exploiting NO$_3^-$. Salt stress might represent an extreme condition, where, in addition to the inhibition of LR elongation, the increased concentration of Na$^+$ ions could interfere with NO$_3^-$ for root uptake sites (Sagi et al., 1997). Under these circumstances, integration of the salt SIMR and NO$_3^-$ signalling pathways leading to a massive increase in LR proliferation would facilitate exploitation of mobile nutrients, thereby allowing the plant to compete better for resources under sub-optimal soil conditions. Furthermore, seedlings (upon which the present work is based), are highly vulnerable to such conditions, which can have severe effects upon seedling growth and establishment. Indeed, the seedling stage has been considered as a bottleneck in a species’ life history (Leck et al., 2008). Seedling responses such as an SIMR could aid in seedling survival and establishment, which ultimately underlies the sustainability of plant communities.

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