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ORIGINAL ARTICLE

Abscisic acid is involved in several processes associated with root system architecture in maize

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Abstract

Studies concerning abscisic acid (ABA) involvement in root system architecture (RSA) and the interaction of ABA with auxin have reported contrasting results. In this study, the effects of exogenous ABA application and withdrawal as well as a combined treatment of ABA with the synthetic auxin 1-naphthaleneacetic acid (NAA) were thoroughly investigated in maize. The results showed that ABA reduced both the primary root (PR) elongation and the lateral root density (LRD), whereas NAA inhibited PR elongation but increased LRD. The combined treatment involving ABA and NAA inhibited PR elongation. Regarding ABA withdrawal, PR elongation was restored when ABA was removed from the growth media, but LRD was not restored after ABA withdrawal. However, the results of the combined treatment showed that auxin can reverse the inhibitory effect of ABA on LRD. A more in-depth analysis revealed that the inhibitory effect of ABA on lateral root (LR) formation depends on the stage of development. Exogenously added ABA blocked the development of lateral root primordia (LRPs) in the early stages, but was unable to inhibit the elongation of developed LRPs. These results suggest that ABA arrests the formation of LRPs rather than the growth and emergence of LRPs and their subsequent elongation.

Keywords Abscisic acid · Auxin · Root system architecture · Maize

Introduction

Phytohormones regulate plant growth by controlling a developmental programme that provides great plasticity allowing root architecture to adapt to changing environmental conditions (Davies 2010). Root system architecture (RSA) includes components such as primary root (PR) elongation and recurrent ramification leading to lateral root (LR) formation (Márquez et al. 2016). Therefore, the extension of RSA depends on the lateral root density (LRD) as well as the elongation of the PR and LRs (Duan et al. 2013).

LR formation involves several developmental events (Péret et al. 2009; De Rybel et al. 2010). First, pericycle cells are primed to become founder cells, after which they form lateral root primordia (LRPs) by asymmetrical division through lateral root initiation (LRI). Next, the LRPs grow through the root cortex and emerge from the PR, forming lateral roots in which a new meristem is activated and causing subsequent root elongation (Torres-Martínez et al. 2019).

PR elongation and LR formation are complex developmental processes involving the participation of phytohormones, such as auxin, cytokinin (Márquez et al. 2019), abscisic acid (ABA), ethylene, salicylic acid, and jasmonic acid (Deak and Malamy 2005; Péret et al. 2009; Ubeda-Tomás et al. 2012).

Auxin has been proposed to be the main hormone controlling both PR elongation and LR formation (Fukaki and Tasaka 2009; Du and Scheres 2018; Alarcón et al. 2019). It is well known that the external application of auxin inhibits PR and LR elongation and increases LR density of maize roots (Márquez et al. 2016), which is a manifestation of stress-induced morphogenic responses (Potters et al. 2007). LR formation involves sequential steps that require auxin involvement (Lavenus et al. 2013; Du and Scheres 2018).

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Pericycle cells are primed, and the initial phase in which these cells become founder cells requires auxin accumulation (Moreno-Risueño et al. 2011). Then, the presence of auxin is also essential for asymmetric divisions conducting to the formation of LRPs (De Smet 2007). The growth of LRPs through the root cortex and their emergence to become LRs also require auxin, which in this case originates from the aerial parts of seedlings (Bhalerao et al. 2002). This indicates that the auxin source changes in the developmental phases of LR formation (Du and Scheres 2018). Auxin is considered the key hormone in LR development (Nibau et al. 2008), and other plant hormones interact with auxin to shape root architecture in response to environmental change (Harris 2015). The antagonistic effects of auxin, ABA, and cytokinin on LR formation are well known, with auxin being a stimulator, whereas ABA and cytokinin are inhibitors (Signora et al. 2001; Fukaki and Tasaka 2009). In maize, it has been shown that exogenous auxin enhances LR density (Alarcón et al. 2019), while cytokinin reduces LR density (Márquez et al. 2019).

The role of ABA under water stress conditions has also been widely reported, as ABA can maintain root elongation under low water potentials (Saab et al. 1990), while high concentrations of ABA reduce PR elongation (Li et al. 2017). ABA signalling in roots is required for a variety of processes, such as the maintenance of PR elongation and the repression of LR formation under water-deficit conditions (Sharp et al. 2004; Deak and Malamy 2005). High concentrations of ABA have been reported to inhibit the growth of both lateral and primary roots. However, such inhibitory effects are much stronger on LRs than on PRs, suggesting that different signalling mechanisms are at play in both types of roots. LR growth is inhibited by ABA in response to salt stress (Duan et al. 2013) and water stress (Signora et al. 2001; De Smet 2003). Concerning salt stress, the proximal region of LRs elongated in the absence of NaCl was not affected, but the distal region of LRs growing in the presence of NaCl decreased. In this context, ABA can stimulate growth of PRs as well as inhibit growth of LRs via signalling within the endodermal layer (Duan et al. 2013).

Phytohormone interactions during LR formation have been reported to occur (Fukaki and Tasaka 2009). Among these phytohormones, ABA has been shown to interact with auxin in response to biotic or abiotic stresses (De Smet et al. 2006), mainly in opposing ways (Sun and Li 2014), and links between auxin and ABA have been observed. Root elongation is inhibited by both ABA and auxin at high concentrations (Thole et al. 2014; Márquez et al. 2016). Exogenous ABA at a low concentration (0.1 µM) enhances root growth, but at a higher concentration (10 µM), it inhibits root growth. Auxin can stimulate the inhibitory effects of ABA on germination (Brady et al. 2003), and ABA sensitivity of certain auxin-insensitive mutants, such as *axr1* and *axr2*, was also decreased in terms of germination and root elongation inhibition (Wilson et al. 1990). ABA inhibits the development of RSA by altering several processes that affect auxin-related actions. ABA at high concentrations reduces the expression of genes related to auxin transport (AUX1, PIN1, PIN3, PIN5, and PIN7) in roots by lowering auxin levels, resulting in root growth inhibition (Sun et al. 2018). Compared with wild-type Arabidopsis, the Arabidopsis mutants *aux1* and *aux4* show less sensitivity to ABA inhibition of PR elongation (Rowe et al. 2016; Li et al. 2017). Auxin promotion of LR formation counteracts the effects of ABA, as plants overexpressing ABSCISIC ACID INSENSITIVE 4 (ABI4) show a reduced number of LRs, whereas the abi4 mutant presented increased the number of LRs. In addition, ABI4 expression reduced the expression of the auxin-efflux carrier PIN1. Mutants defective in auxin transport or signalling exhibit resistance to the effects of ABA on root elongation inhibition, showing that auxin transport is required for ABA inhibition (Wang et al. 2011). However, auxin inhibition in root elongation does not need ABA signalling. Therefore, these results suggest that auxin works downstream of ABA in root elongation. Recently, it was found that exogenous ABA reduces the expression of PIN2 an auxin transporter (Xie et al. 2021).

Auxin is required for a number of stages of LR development. In fact, it has been proposed that the lateral root initiation (LRI) is dependent on an auxin source located in the root apex, but regarding LR emergence, indole-3-acetic acid (IAA) transport from the shoots to the roots is crucial (Bhalerao et al. 2002). ABA-auxin interactions in LR formation have been recently reviewed (Emenecker and Strader 2020). ABA has been shown to be an inhibitor of LR formation in specific developmental stages: immediately after emergence and before the activation of the meristem of the new LR (De Smet et al. 2003). The inhibition of LR elongation is mediated by reducing polar auxin transport, suggesting the counteraction of auxin with ABA and cytokinin in regulating LR development (Shkolnik-Inbar and Bar-Zvi 2010). The ABA-insensitive mutant abi4 presents increased LRD, and ABI4 expression is enhanced by ABA and cytokinin but repressed by auxin. Exogenous auxin is more effectively transported in abi4 roots than in wild-type roots, and ABI4 inhibits both the initiation and elongation of emerged LRs. Although ABI4 affects several stages of LR formation, its effect is greater on LRP initiation than on elongation of emerged LRs (Shkolnik-Inbar and Bar-Zvi 2010). In maize, the inhibitory effect of cytokinins on LR development has been shown to take place during the earliest stages of LRP initiation (Márquez et al. 2018).

In addition, mutants defective in proteins related to auxin transport and gravity responses (Rashotte et al. 2001) have decreased sensitivity to ABA. Moreover, ABA inhibits the expression of the auxin-responsive reporter gene *DR5*-GUS

in emerging LRs (De Smet et al. 2003), and the *abi3* mutant, originally isolated for its ABA insensitivity during germination, exhibits little sensitivity to LR stimulation to auxin (Brady et al. 2003). The expression of *ABI3* is induced by auxin in the roots (Brady et al. 2003) when its maize *ABI3* homologue *VP1* is expressed in Arabidopsis. ABA can suppress LR formation induced by auxin (Suzuki et al. 2001). These facts suggest that ABI3 is an intermediary of auxin-ABA interactions. An Arabidopsis mutant defective in the indole-3-butyric acid response, ibr5, showed less sensitivity to IAA and ABA than the wild type. Also, when compared with the wild type, this mutant exhibits longer PRs but fewer LRs that elongated to a lesser degree. It has been proposed that IBR5 is a phosphatase that connects auxin and ABA signalling pathways (Monroe-Augustus 2003). However, contrasting results have been found after ABA application in rice: ABA unexpectedly promotes LR growth in rice. Moreover, exogenous ABA partly compensates for deficiencies in the low levels of ABA in mutants that also exhibit inhibited PR and LR formation (Chen et al. 2006).

In summary, ABA is involved in PR growth and several phases of LR development. The results in rice suggest that monocotyledonous and dicotyledonous plants could exhibit different ABA behaviours. Therefore, the objective of this work was to determine the involvement of ABA in RSA development of maize seedlings through LR formation via analysis of the three main phases that occur at several distances from the root apex in a single root along the longitudinal root axis: (i) initiation and LRP development, (ii) emergence of LRPs, and (iii) LR elongation. In addition, ABA–auxin interactions were analysed.

Materials and methods

This experiment has been performed essentially following the methodology reported in previous works (Márquez et al, 2019). Maize (cv. DK 6664) seeds were washed twice under running tap water for 15 min and then imbibed in distilled water with aeration at 30 °C for 24 h. During this period, the seeds developed radicles that were approximately 1 mm in length, after which they were placed on wet filter paper in plastic boxes and covered with filter paper. The boxes containing the seeds were maintained vertically in darkness at 30 °C, and after 24 h, roots elongated to a length of 30 ± 5 mm. Discs with ten chosen seedlings with uniform root length were placed in bottles containing 1.35 L of growth media composed of 1 mM HEPES, 1 mM $CaCl_2$, and 10 mM KCl (pH 6.0) at 30 °C in darkness. The growth media were aerated using an aquarium pump. After 24 h, the PRs were approximately 80–90 mm long, and treatments were applied by adding small volumes of concentrated solutions to the growth media. The root lengths were measured using a ruler (precision of ± 1 mm) individually before and 24, 48, and 72 h after applying the phytohormones. The application of such hormones originated two consecutive root regions, corresponding to the segments which elongated before and after the treatment. The proximal region (approximately 6 cm), grown before the auxin application, was further divided into two different zones. The criteria for such a division were the presence of detectable LRPs. The basal zone corresponded to the oldest segment presenting LRPs when the hormone was applied, whereas the apical zone did not present any detectable LRPs when ABA was applied. The portion of roots that elongated within 24/48 h after the treatment was denoted as the distal region (Márquez et al. 2019). In experiments where roots were transferred from a medium with auxin to a new hormone-free medium, the roots were washed by submerging them twice in 1 mM HEPES and 1 mM $CaCl_2$ before being placed in the new medium.

The roots were allowed to grow for 24, 48, or 72 h to allow LRPs to develop LRs and thus determine the evolution of the primordia and facilitate LR quantification after treatment. Then, the PR length was measured, and several root zones were chosen. These zones were fixed and cleared in FAA solution [50% (v/v) ethanol + 36% (m/v) formaldehyde + 100% acetic acid, 91:6:3] during 48 h. Afterwards, the roots were transferred to 70% ethanol for 48 h prior to LR quantification. Several 1 cm-long root segments were imaged, and LRPs were counted. The LRD was expressed as the numbers of LRs and LRPs per centimetre. In the distal region where LRs are not usually observed, LRPs were counted under a dissecting microscope. LR length was measured for root segment images using ImageJ.

The data represent the mean ± standard deviation of 20 seedlings per treatment. The experiments were repeated at least twice independently. After Kolmogorov–Smirnov's test for normality was performed, comparisons between the media were performed by Student's *t* test or ANOVA followed by Tukey's test at $P < 0.05$ using SPSS v. 21.0.

Results

Effects of exogenous abscisic acid on maize primary root elongation

Exogenous ABA inhibited PR elongation in a concentration-dependent manner (Fig. 1). Under our experimental conditions, an extended range of ABA concentrations was applied to analyse the effects on RSA. Only concentrations higher than 0.01 µM significantly reduced the length of roots of maize growing in a hydroponic system, whereas ABA applied at a concentration of 0.01 µM did not significantly affect PR elongation. The increases in length were measured

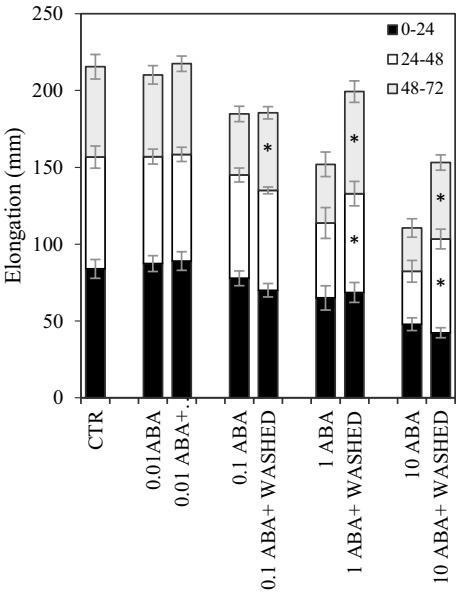


Fig. 1 Abscisic acid (ABA) inhibited primary root elongation in maize. Roots of 80–100 mm in length were individually measured, and then, ABA was applied to the growth media at the indicated concentrations. Then, roots were measured again after 24, 48, and 72 h. Roots treated for 24 h with ABA were transferred to a new fresh medium ABA-free and elongation was measured at the same times. Data represent mean ± standard error, $n = 20$. Asterisks indicate significant differences between non-transferred and transferred roots treated with the same ABA concentration at the same times (Student´s test, $P < 0.05$, $n = 20$)

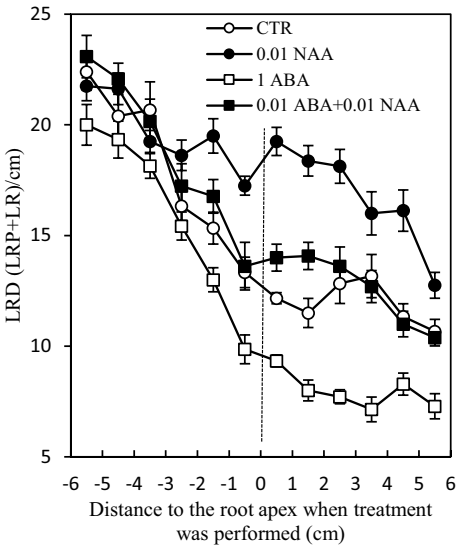


Fig. 2 Abscisic acid (ABA) reduced lateral root density (LRD) and naphthalene acetic acid (NAA) reversed its inhibitory effect in proximal and distal region. Roots of 80–100 mm in length were grown with 1 µM ABA alone or combined with 0.01 µM NAA for 48 h. The point 0 was the root apex when treatment was performed. Both regions have been divided in 1 cm segments and the values of LRD in each segment were assigned to the medial point. Values represent mean ± standard deviations, $n = 20$

during three consecutive periods of 24 h. The elongation of the untreated roots was higher in the first period (0–24 h) when the roots had an initial length of 85–100 mm. The elongation on the second (24–48 h) and third days (48–72 h) was 87% and 70%, respectively, compared to that on the first day. This effect was also observed in the ABA treatments, but the reduction was higher than in untreated roots than in the treated roots as the concentration increased. When 10 µM was applied, the elongation in the period from 24 to 48 h represented 70% and in the period from 48 to 72 h represented 60% of the elongation in the first period at the same concentration.

Abscisic acid withdrawal rescued the inhibitory effect on primary root elongation

To determine whether presence of ABA in the growth medium is required for maintaining inhibition of root elongation, roots were subjected to ABA for 24 h, after which they washed. The seedlings were then transferred to ABA-free media where they were allowed to grow for two more consecutive periods of 24 h. After ABA application, the root elongation rate was reduced depending on the ABA concentration. When ABA was withdrawn from the growth media, the elongation rate of the roots recovered, reaching values similar to those of the control roots (Fig. 1). Recovery was complete when ABA was applied at 1 µM or less. However, when 10 µM ABA was added, a strong inhibition was detected, in which the root elongation was reduced by 50% in the period from 0 to 24 h. When the roots treated with 10 µM ABA, washed, and then the seedlings were transferred to new media without ABA, root elongation recovered, albeit incompletely, reaching up to 84% of the elongation of the untreated roots. Significant differences between the elongation rates of roots growing in the presence of ABA and after ABA withdrawal were found at concentrations up to 0.1 µM ABA (Student's *t* test, $P < 0.05$). Therefore, the continuous presence of ABA is required for PR inhibition.

Abscisic acid reduced lateral root density

Exogenous ABA reduced LRD along the primary maize root, and this effect depended on the concentration. The maximal inhibition was observed in the apical zone of the proximal region located at the root apex when the treatment was applied. As an example, ABA at concentrations up to 1 µM caused a strong decrease in the top 2 cm of the apical region (Fig. 2). However, in the basal zone of the proximal region located more than 3 cm from the root apex, no notable differences in LRD were found between untreated and ABA-treated roots at any ABA concentration.

Since the LRPs are in different stages of development in the proximal zone, we imaged sections of the basal and apical zones of the proximal region to analyse the stages of the LRPs in these zones. In Table 1, the LRPs of several 1 cm segments in the proximal region are shown, indicating that LRs are formed acropetally. In previous reports, the proximal region that elongated before treatments were applied is referred to as the proximal region but lately has been divided into two zones, i.e., apical and distal ones (Márquez et al. 2019), according to the presence of LRP. LRPs are detectable at some distance from the apex, and LRP density increases with increasing distance from the apex. In these experiments, the first LRP was detected at 1.85 ± 0.14 mm from the root apex, which is in accordance with the findings of previous experiments in maize (Márquez et al. 2019). LRPs were counted along several segments of the PR according to the distance from the root apex and the LRP stage when treatments were applied (Table 1). As expected, the developmental stage of LRPs was greater in the more basal regions compared with the more apical regions. First, LRs were not detected along the entire proximal segment when treatments were applied. This indicates that emergence has not yet occurred, and the LRPs differ on the basis of their growth through the PR cortex. As shown in the images in Table 1, the LRPs on segment − 1.5 are almost imperceptible, while on segment − 4.5, they are easily detectable. In summary, a strong ABA inhibitory effect on LRD was observed in the most apical 1 cm segment that had no LRPs when ABA was applied. As expected, the developmental stage of LRPs was greater in more basal regions than in more apical ones. The first LRPs detectable in the apical zone were in the initial phase, as they are almost imperceptible, whereas the LRPs located in the distal zone are easily detectable at 4 cm from the root apex (Table 1). In the distal region, LRD was also reduced by ABA in a dependent-concentration manner (Fig. 2).

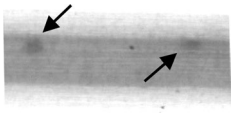
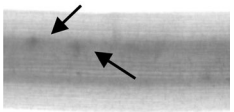
To perform a statistical analysis, the LRP data were quantified in the two most apical and basal cm of the proximal and distal regions (Table 2). The data clearly show that the inhibitory effect significantly increased with increasing concentration in both regions.

Table 2 Lateral root density (lateral roots + lateral root primordium), LR + LRP in segments of 2 cm located at proximal and distal regions

Treatment	Segment		Elongation (mm/48 h)
	2 cm Proximal	2 cm Distal	
CTR	29.0 b	26.0 bc	143.1 a
0.01 NAA	36.7 ab	37.6 a	105.9 b
0.01 ABA	29.4b	24.4 bc	145.2 a
0.01 ABA + 0.01 NAA	39.1 a	36.7 a	100.5 b
0.1 ABA	28.3 b	20.9 de	142.4 a
0.1 ABA + 0.01 NAA	34.7 a	30.5 ab	91.4 bc
1 ABA	22.8 c	17.3 ef	92.1 bc
1 ABA + 0.01 NAA	21.8 c	23.9 cd	72.7 de
2.5 ABA	20.8 c	16.0 ef	80.6 cd
2.5 ABA + 0.01 NAA	22.8 c	23.5 cd	57.8 ef
5 ABA	20.3 c	16.0 ef	59.6 ef
5 ABA + 0.01 NAA	19.6 c	18.1 ef	46.7 fg

Numbers represent the mean of the total LR + LRP counted in 2 cm of the most apical zone of the proximal region and the oldest segment of the distal region formed before and after hormone treatment, respectively. Elongation represents the mean of the increment in root length in 48 h after treatment ($n = 10$ roots). Different letters indicate significant differences between treatment in the same segment or elongation (ANOVA and Tukey's test, $P < 0.05$)

Table 1 Lateral root primordium density (LRPD) and developmental stages of (lateral root primordium) LRP along the proximal region of the primary root when treatment was performed

	Proximal region					
	Distal zone			Apical zone		
	Distance from the root apex (cm)					
	− 5.5	− 4.5	− 3.5	− 2.5	− 1.5	− 0.5
LRPD	18 ± 3	15 ± 2	14 ± 2	12 ± 2	3 ± 2	–
						

Data represent mean ± standard deviation of $n = 10$. Photographs show segments located at 1.85 cm that corresponds with the distance where the first LRP is detected (right) and 4.5 cm (left) from the root apex, respectively

Exogenous auxin reversed the abscisic acid inhibitory effect on lateral root density

Before we analysed the effects of ABA–auxin interactions on LR formation and development, the effects of the combined ABA-1-naphthaleneacetic acid (NAA) treatment on PR elongation were assessed. When applied at 0.01 µM, NAA also reduced PR elongation. The combined treatment of ABA and NAA showed a cooperative effect on the reduction of root elongation (Table 2), since NAA at all concentrations increased the inhibitory effect of ABA.

Regarding LRD, the most sensitive segments to hormones were those elongated just before and after the treatments were applied. The effects of several ABA concentrations on LRD, either applied alone or in combination with 0.01 µM NAA in the most apical 2 cm of the proximal region and most distal 2 cm of the distal region of the PR, are presented in Table 2 together with the PR elongation within 48 h under the different treatments. As expected, NAA increased the LRD for both segments, showing the stimulatory activity of auxin in LR formation. The stimulatory activity was 26% for the proximal segment, but increased to 44% for the distal segment. These results are in agreement with those of previous reports in maize (Alarcón et al. 2019). When ABA was applied at 0.01 µM, no effects were measured on either LRD or elongation. Moreover, this concentration did not change the effect of 0.01 µM NAA on LRD or elongation. LRD decreased as ABA concentration increased. Regarding elongation, NAA was not able to reverse the inhibition caused by ABA, but a cooperative effect on elongation between ABA and NAA was recorded.

Abscisic acid withdrawal did not rescue the inhibitory effect on LR density

LRD is mainly affected by ABA with the top two 2 cm of both the apical zone of the proximal region and the distal region. To determine whether the ABA inhibitory effect could be reversed by ABA withdrawal, the roots were incubated for 24 h with ABA and then washed, after which the seedlings were transferred new ABA-free media where they were allowed to grow for another 48 h. LRD was quantified in washed and control roots treated with 1 and 10 M ABA. The results showed that inhibitory action increased with ABA concentration, but no significant differences in LRD between the roots continuously grown in the presence of ABA and the washed roots were found (ANOVA and Tukey's test, $P < 0.05$) for the same concentration of ABA (Table 3). This suggests that ABA inhibition takes place in the earliest stages of LRP formation and that once the effect occurs, pericycle cells are not able to recover their ability to initiate LRPs.

Table 3 Effect of abscisic acid (ABA) withdrawal on lateral root density

Treatment µM	Segment	
	2 cm Proximal	2 cm Distal
CTR	33.8 a	28.6 a
1 ABA	28.8 b	22.5 b
1 ABA + Washed	29.6 ab	20.0 b
10 ABA	18.9 c	15.3 c
10 ABA + Washed	14.5 c	15.3 c

Numbers represent the mean of the total LR + LRP counted in 2 cm of the most apical zone of the proximal region and the oldest segment of the distal region formed before and after each treatment ($n = 10$ roots). Different letters indicate significant differences between treatments in the same segment (ANOVA and Tukey's test, $P < 0.05$)

Abscisic acid inhibited lateral root elongation

ABA differentially inhibited the promotion of LRD in the proximal region, causing greater amounts of inhibition when LRPs are in the early stages of development. LRD decreased in the apical zone, but no important effects on LRD were detected in the basal zone, where LRPs were more developed when ABA was applied (Fig. 2, Table 1). Previously, to study the effects of ABA on LR elongation, we analysed the developmental stage of LRPs along the proximal region before ABA application (Table 1). The effect of ABA on LR elongation was analysed within a segment of the proximal zone located − 2.5 cm from the root apex when ABA was applied; for this, we measured LR length after 24, 48, and 72 h of treatment.

The length of untreated PRs increased by 133 ± 6.2 mm 48 h compared with the initial length (85–100 mm, $t = 0$ h) (Fig. 1), and LRPs emerged and led to the formation of LRs along the entire proximal region (Fig. 3). LR development was inhibited by ABA, and the inhibition was significant at concentrations up to 1 µM (Fig. 3). LR length was measured for segments of 1 cm located at − 2.5 cm from the apex to quantify LR elongation (Table 4), and it was smaller as the ABA concentration increased. At 10 µM ABA, primary lateral roots (PRLs) were shown to be unable to emerge by 48 h. In the following 24 h (48–72 h period), LRs of untreated seedlings elongated by 5.5 ± 1.8 mm, and the increase in length was inhibited with increasing ABA concentration. At 72 h after ABA application, the roots incubated with 10 µM ABA presented LRs with a length of 0.50 ± 0.15 mm. Similar results were observed in more proximal segments.

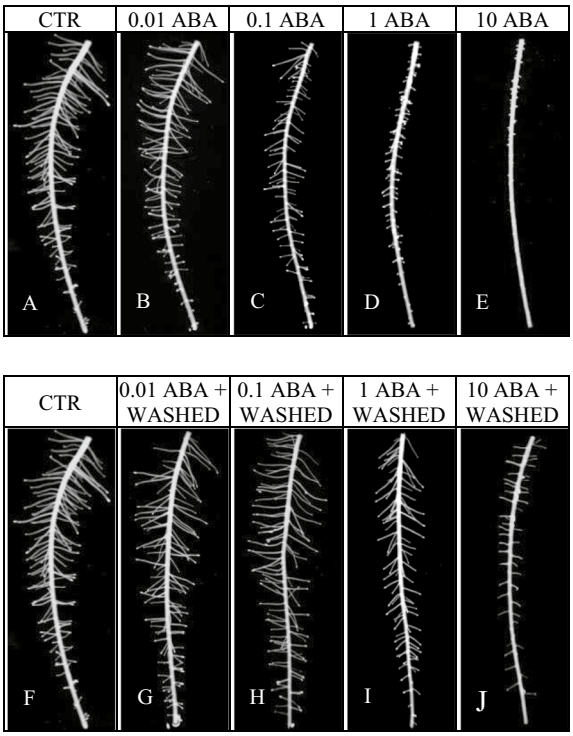


Fig. 3 Effect of abscisic acid (ABA) and ABA withdrawal on lateral root (LR) development. Roots of 80–100 mm length were incubated under several ABA concentrations for 72 h (**A**–**E**). ABA is removed from the incubation medium after 24 h and roots were grown in an ABA-free medium for 48 h more (**F**–**J**). The root segments represent 6 cm of the proximal region that elongated before ABA application

Table 4 Lateral root length in 1 cm segment located at − 2.5 cm from the root apex in the proximal region

[ABA] µM	ABA 48 h	ABA 72 h	ABA 24 h –ABA 24 h	ABA 24 h –ABA 48 h
0	2.72 a	8.31 b	–	–
0.01	2.37 a	6.80 c	5.01 b	7.00 c
0.1	2.43 a	3.35 b	4.0 b	5.24 c
1	1.12 a	1.84 b	2.88 c	5.22 d
10	0.00 a	0.50 b	1.69 c	2.12 d

Roots were grown at several conditions: continuously with abscisic acid (ABA) for 48 and 72 h, 24 h with ABA and then transferred to a medium ABA-free and grown for 24 or 48 h more. Elongation represents means of lateral root lengths in mm at the end of indicate period ($n = 10$ roots). Different letters indicate significant differences between treatments for the same ABA concentration (ANOVA and Tukey's test, $P < 0.05$)

Abscisic acid withdrawal rescued the inhibitory effect on lateral root development

To determine whether the presence of ABA is also required to inhibit LR elongation, we evaluated LR development along the PR after the withdrawal of ABA at several different concentrations (Fig. 3). The roots were grown in the presence of several different ABA concentrations for 24 h; afterwards, the seedlings were separated into two groups and grown under different conditions: in one group, the roots were grown for another 24 or 48 h in the presence of ABA, and in the other group, the seedlings were transferred to fresh ABA-free media. The LRs were longer after ABA withdrawal (Table 4) (Student's t test, $P < 0.05$, $n > 30$). In addition, an increase in LR length between 24 and 48 h after ABA withdrawal was detected. We noted that 10 µM ABA arrested any increase in PLR, as no LRs were observed after 48 h, but LRs of 1.69 ± 0.60 in length were measured for the roots of seedlings transferred to ABA-free media after 24 h of incubation with ABA (Table 4). However, small LRs were detected after 72 h in roots subjected to 10 µM ABA.

Lateral root density was not restored after abscisic acid withdrawal, but lateral root primordia developed into lateral roots

Finally, we analysed whether LRPs formed within the ABA inhibitory-effect area were able to grow and develop new LRs, as well as whether new LRs were formed on these segments where LRI was arrested by ABA application. LRPs under several experimental conditions are presented in Fig. 4. As has already been shown, LRPs were at a more advance developmental stage in more basal regions compared with apical regions (Table 1), and the evolution of LRPs was evaluated in two regions: the most apical 2 cm segment and the segment − 2.5 cm from the root apex of the proximal region. The control roots exhibited LRs at 48 h on both segments, and as expected, the LRs were longer on the more basal segment than in the more apical one. Moreover, the LR length increased between 48 and 72 h for both segments. When the roots were grown in 10 µM ABA, LR development was blocked; in most apical segments, LRPs were detected after 72 h, but no emerged LRs were detected. However, in the − 2.5 cm segment, LRPs increased in size after 48 h, and LRs had emerged at 72 h. When the seedlings were transferred after 24 h of treatment with 10 µM ABA to ABA-free media, we observed that LRs emerged after 48 h, and an increase in LR length was measured at 72 h. Different LR densities were observed for the two selected segments. As expected, LRs were longer in the most basal segment. In addition, in the most apical segment where no LRPs had formed when ABA was applied, only a few LRs were detected compared to the number along the − 2.5 cm segment. Taken together, these results indicate that ABA blocks the early stages of LRP formation rather than the growth and emergence of LRPs and subsequent LR elongation.

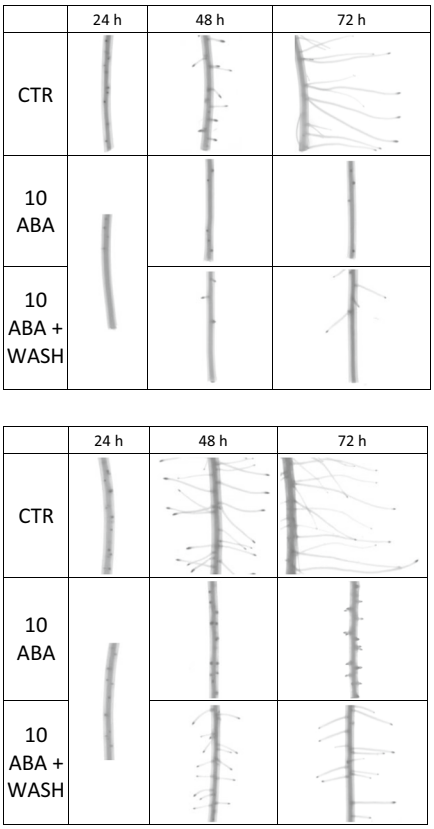


Fig. 4 Abscisic acid (ABA) reduced LR elongation. Roots of 80–100 mm were grown in different media for 72 h. Control roots (CTR) were grown without ABA, while ABA-treated roots were incubated for 24 h under 10 µM ABA and then divided in two groups. One of them continued growing in the presence of ABA and the other was transferred to an ABA-free medium. After that, roots from both groups were grown for 48 h more. Photographs show the most apical cm of proximal region (top) and the segment located at − 2.5 cm from root apex (bottom) when treatment was applied at the indicated times

Abscisic acid differentially inhibited lateral root and primary root elongation

ABA inhibited both PR and LR growth in a concentration-dependent manner. The inhibition of PR and LR elongation in response to ABA increased with time, but was greater for LR. The results in Table 5 clearly show that PRs were less sensitive to ABA than LRs were. High concentrations of ABA (10 µM) reduced root elongation by 48–52% at 48 and 72 h, respectively; however, but this concentration completely blocked emergence of LRPs at 48 h, and only small LRs were observed at 72 h (Table 4). ABA at 1 µM reduced PR elongation by only 30%, whereas LR elongation was reduced by 60% at 48 h, and this difference was greater at 72 h.

Table 5 Differential effect of abscisic acid (ABA) in primary root (PR) and lateral root (LR)

	Primary root (%)		Lateral root (%)	
[ABA] µM	0-48 h	48-72 h	0-48 h	48-72 h
0	100.0	100.0	100.0	100.0
0.01	100.0	90.5	87.1	79.3
0.1	92.4	67.6	89.3	16.5
1	72.5	64.9	41.2	12.9
10	52.4	47.9	0.0	8.9

Increase in root length is expressed as percentage of the untreated roots (100%) at the indicated times. PR length is presented in Fig. 1 and LR length in 1 cm segment located at -2.5 cm from the root apex in the proximal region is presented in Table 4

Discussion

Abscisic acid inhibits lateral root development at several different stages

ABA has been reported to stimulate and inhibit root growth depending on the root type and species as well as environmental and experimental conditions that may be amenable to complex interactions of auxin and ABA in the regulation of RSA (Li et al. 2017). ABA inhibits LR development in Arabidopsis (De Smet et al. 2003), and ABA accumulation in response to salt stress inhibits LRI and development in maize (Lu et al. 2019). Although ABA is an inhibitor of root growth under well-watered conditions, ABA synthesis is required to maintain root growth under water stress (Saab et al. 1990). In contrast, in rice, ABA promotes LRI (Chen et al. 2006).

In this study, we analysed the effects of ABA on several manifestations of maize RSA both by reversing ABA inhibition via withdrawal and by applying exogenous auxin. RSA is a result of several processes, such as PR elongation, LRP initiation, emergence, and LR elongation (Péret et al. 2009; De Rybel et al. 2010; Torres-Martínez et al. 2019). Consequently, we measured parameters such as LRD, PR, and LR elongation and the emergence of LRP through the overlying cell layer.

Abscisic acid differentially inhibits primary root and lateral root elongation

PR elongation is reduced by ABA in a concentration-dependent manner (Fig. 1), indicating that changes in environmental conditions in which increased ABA levels result in a reduction in PR length. It is well known that ABA is involved in the response to both water and salt stress (Duan et al. 2013). In our experiments, ABA inhibited PR elongation, but ABA withdrawal rescued the elongation rate. A

similar behaviour was observed for LRs; the inhibition of LR elongation by ABA was rescued when roots were transferred a new media without ABA (Fig. 1). Therefore, ABA could be considered a mediator of external conditions to allow roots to grow and adapt to environmental changes (Harris 2015).

In maize, although ABA inhibited both LR and PR growth, LR elongation was more severely affected than was PR elongation (Table 5). These findings are in accordance with those of previous reports that showed that ABA causes a stronger reduction in growth in LRs than in PRs, indicating that hormone sensitivity differs in these two kinds of roots (Duan et al. 2013; Xing et al. 2016). These results are in agreement with those reported in Arabidopsis, where it has been proposed that ABA suppresses the activity of new LR meristems, which are put in a reversible dormant stage that can be activated by the withdrawal of ABA. PRs were not affected by ABA at 10 µM, but 0.01 µM reduced LRD by 80% (De Smet 2003).

Abscisic acid inhibits lateral root initiation and elongation

The results indicate that ABA is involved in LRI and elongation in different ways. Once LRPs are formed, since ABA only delays LR elongation, but does not completely block LR development, the effects on LRI were measured by quantification of the LRD at 48 h after ABA application (Table 2). Exogenous ABA reduced LRD along the root segment where LRI takes place, indicating that this process is affected by ABA. This inhibition was not reversed by ABA withdrawal, which implies that this zone is the most sensitive to LRI. At 48 h after withdrawal, ABA permanently blocked LRI, and no new LRs were formed (Fig. 4). These results suggest that the critical point of ABA action is LRI and that once LRPs are formed, ABA can control LR elongation only by via changes in ABA concentration. It has been proposed that LRI is an auxin-dependent process and that alterations in auxin transport by ABA could disrupt the auxin gradient and arrest LRI (Shkolnik-Inbar and Bar-Zvi 2010). In addition, LRI seems to be restricted to a certain developmental stage, and once roots overcome this stage, they lose the ability to initiate new LRs. The results in Fig. 4 are in partial agreement with those in Arabidopsis: when seedlings that were grown in the presence of ABA and whose roots had shown arrested LR were transferred to new ABA-free media, LR elongation was restored, indicating that LR elongation in response to ABA is reversible. However, LRI was not affected by ABA, as a similar LRD was detected between control roots and 0.5 µM ABA-treated roots (De Smet et al. 2003). Other research has reported that ABA at 1 µM is sufficient to block the development of visible LRs in Arabidopsis (Harris 2015). However, in maize, higher concentrations (10 µM) are required to totally prevent LR elongation (Table 4).

Abscisic acid–auxin interactions

In maize, PR and LR elongation is inhibited by ABA and auxin (Table 5), in accordance with the findings of a previous report showing that root elongation is inhibited by both ABA and auxin at high concentrations (Thole et al. 2014), but ABA interacts with auxin in contrasting ways (Sun and Li 2014). The results of this work showed that auxin stimulated LR formation, while ABA inhibited LR formation. Auxin stimulates but ABA inhibits LR formation. Moreover, PR inhibition by ABA requires intact auxin transport and signalling, whereas LR elongation does not (Emenecker and Strader 2020). The interaction between ABA and auxin in RSA is complex, as auxin polar transport is responsible for auxin movement in plants, and ABA alters the expression of auxin transporters (Brady et al. 2003; Sun et al. 2018; Xie et al. 2021). Moreover, ABA interferes with auxin transport. Mutants identified as being auxin-insensitive also show decreased ABA sensitivity in terms of root elongation inhibition (Wilson et al. 1990). Indeed, ABA reduces the expression of genes related to auxin transport in the roots, resulting in a decrease in auxin levels and root growth inhibition (Sun et al. 2018). In addition, compared with wild-type Arabidopsis plants, Arabidopsis mutants defective in auxin show less sensitivity to ABA inhibition of PR elongation (Rowe et al. 2016; Li et al. 2017).

Differences in the ability to reverse the inhibitory effect of ABA on RSA were found between natural and synthetic auxin. Both natural IAA and synthetic NAA (both of which are auxins) can restore LR phenotypes in Arabidopsis, and an inhibitor of auxin polar transport 1-naphthylphthalamic acid, NPA, was shown to reduce LRD in maize. IAA restores the ABA effect on elongation, but does not rescue the inhibitory action of ABA in LRI (Lu et al. 2019). However, in our experiment, when applied in combination with ABA, the synthetic auxin NAA restored LRD (Fig. 2, Table 2). NAA can enter cells without a transporter, whereas the natural auxin IAA requires specific transporters. Regarding LRs, ABI4 plants overexpressing ABA showed a reduced number of LRs, whereas abi4 mutants presented increased numbers of LRs. In addition, ABI4 expression reduces the expression of the auxin-efflux carrier PIN1 (Shkolnik-Inbar and Bar-Zvi, 2010). Therefore, NAA can accumulate in cells to initiate LRPs even when IAA transport is affected by the presence of ABA. Once LRPs are formed, ABA can only delay the growth of LRPs and their emergence, since the effects of ABA–auxin interactions on LRI and subsequent development of LRP are different. In addition, the auxin source required in several phases of LR formation is distinct: initiation does not depend on shoot-derived auxin,

whereas this source is essential for growth and emergence (Torres-Martínez et al. 2019). The different effects of auxin on ABA-induced inhibition of RSA could be related to the distinct source of auxin required in several phases. Auxin accumulation in founder cells is required for LRI, and this auxin is not derived from the shoots. However, the growth and emergence of LRPs and LR elongation require auxin transport from the shoots (Reed et al. 1998).

More differences between the effects of NAA and IAA on maize root development were found. IAA at 10 µM only reduced PR elongation and slightly increased LRD (Lu et al. 2019), whereas 0.01 µM NAA reduced PR elongation by 30% and increased LRD by 60%, showing the strong effect of NAA on root growth (Table 2). In Arabidopsis, the application of 0.5 µM ABA reduced LR formation by 68% without affecting PR elongation (De Smet et al. 2003). However, the effects of ABA on rice seedling roots were very different. Instead of blocking LR formation, ABA promoted LR and root hair growth (Chen et al. 2006).

In response to salt stress, elongation of the proximal region in the absence of NaCl was not affected, but the distal region of the PRs of seedlings grown in the presence of NaCl exhibited a reduction in LRs. PRs and LRs were inhibited by salt stress in maize, and ABA accumulated mainly in the LRI zone. ABA–auxin interaction effects on LR formation have been recently reviewed (Emenecker and Strader 2020). The auxin requirements depend on the stage of LR development. The auxin source for LRI is the root apex, whereas IAA transport from the shoots is essential for LR emergence (Bhalerao et al. 2002). ABA inhibits LR formation immediately after LR emergence and before activation of the meristem of the new LR (De Smet et al. 2003). However, it has been shown that LR and PR inhibition occur through different mechanisms (Lu et al. 2019). In our experiments, the main effect of ABA on LRs was observed in the zone where LRP initiation takes place (Fig. 2). LR emergence did not occur after 72 h in zones where initiation had not started when ABA was applied, but LRs emerged in zones where growth of LRPs had previously started (Fig. 4), suggesting that ABA inhibits LRI rather than the growth of LRPs. In accordance with these results, it has been shown that ABI4 affects several stages of LR formation, but the effects of ABI4 are greater on LRP initiation than on elongation of emerged LRs (Shkolnik-Inbar and Bar-Zvi 2010). Moreover, LRI is confined to a specific zone where pericycle cells initiate the development of LRPs. Under our experimental conditions, this zone is restricted to approximately 2 cm of the root apex (Fig. 2). This zone has already been reported to show high sensitivity to hormone treatment (Márquez et al. 2019) and is critical for combinations of ABA and auxin to initiate LR formation in founder cells. Taken together, the results indicate that ABA differentially affects several phases of LR development in maize and also suggest that competitive interactions between auxin and ABA occur at the LRI stage, as auxin reverses LRD inhibition caused by ABA. However, the withdrawal of ABA did not reverse this inhibitory effect, indicating that auxin accumulation is required for LRI. Regarding LR growth through the PR cortex and LR emergence, we showed that ABA blocks LR growth, but ABA withdrawal reverses LR development in this phase. Finally, LR elongation inhibition by ABA decreased as LR length increased. In addition, LR inhibition caused by ABA was also reversed when ABA was removed from the growth media.

Author contribution statement MVA and JS conceived and designed the research. IF and LG performed the experiments. MVA and JS analysed the data. JS wrote the manuscript. All the authors have read and approved the manuscript.

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Declarations

Conflict of interest The authors declare that there no competing interests.

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