Kinetics of maize leaf elongation
IV. Effects of (+)- and (−)-abscisic acid

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Abstract
Abscisic acid (ABA) is involved in many of the responses of plants to environmental stress. This study focuses on the inhibitory effect of ABA on leaf expansion. In addition, the effects of (+)-ABA, the natural form of ABA, were compared to the effects of (−)-ABA. Leaf elongation rates (LER) were measured for the 3rd leaf of maize plants. ABA concentrations were measured by RIA for total ABA and an ELISA specific for (+)-ABA. ABA was added to the hydroponic solution and changes in LER were measured over time. ABA could inhibit LER within 30 min and reached steady-state LER within 4 h. Internal ABA concentrations in the growing zone of the leaf also reached steady-state concentrations after 4 h. This effect of ABA was reversible, because LER was fully restored upon removal of externally applied ABA, and internal concentrations of ABA in the growing zone returned to normal levels, whereas ABA concentrations remained elevated in mature tissue. Thus, steady-state LER was highly correlated with the steady-state internal ABA concentration of the growing zone. ABA inhibited leaf expansion by increasing the apparent cell wall yield threshold; no other growth parameters were affected. The (−)-enantiomer of ABA had much less effect on LER than (+)-ABA when compared upon an external concentration basis. Internal ABA concentrations rationalized the response, showing that (−)-ABA accumulation was very low, most likely due to low uptake rates. From this analysis, it was determined that LER was equally sensitive to internal concentrations of (+)- or (−)-ABA.

Key words: Abscisic acid, maize, leaf elongation.

Introduction
From genes to stomata, (+)-ABA plays an important role in stress physiology (Davies and Jones, 1991; Munns and Cramer, 1996). (+)-ABA concentrations usually increase when the plant is exposed to a stress, particularly stresses which disturb water relations (Munns and Sharp, 1993). Some positive effects that (+)-ABA provides to the plant are reduced stomatal conductance and maintenance of root growth under stress (see reviews by Munns and Cramer, 1996; Munns and Sharp, 1993). In addition, (+)-ABA-induced gene products may play a role in stress tolerance, but to date there is no direct evidence to support this (Chandler and Robertson, 1994).

Under many circumstances, (+)-ABA appears to inhibit plant growth; in particular, ABA often inhibits leaf expansion (Dale, 1988; Dodd and Davies, 1996; Munns and Cramer, 1996; Munns and Sharp, 1993). This is an appropriate strategy for a plant which is exposed to drought, because a reduced leaf surface area will reduce water loss and improve the plant’s chances for survival. On the other hand, the reduction in leaf expansion may not benefit salt-stressed plants (there is usually no shortage of water, at least under irrigated conditions). In fact, the major limitation of growth to moderately salt-stressed maize plants is restriction of leaf expansion (Cramer et al., 1994). The increase of ABA concentration in plants by moderate salt-stress (He and Cramer, 1996) and water stress (Dodd and Davies, 1996; Munns and Sharp, 1993) is correlated with reduced leaf expansion.

In a review of how drought, salinity and temperature limit cell expansion (Cramer and Bowman, 1993), it was found that there were no universal mechanisms of control. The mechanisms of control vary with genotype and the stress imposed. For example, the response of the cell wall...
extensibility to osmotic stress is highly variable and depends on the genotype and the duration of the stress. Whereas, hydraulic conductivity is almost always reduced for all three stresses, but often this effect is secondary to earlier events. In addition, turgor is almost never affected by these stresses in expanding cells. Thus, when one examines how cell expansion is controlled in a particular genotype, one must examine all of the growth parameters of the Lockhart equation (Lockhart, 1965). In this report, this approach is used to describe how (+)-ABA inhibits cell expansion of intact maize leaves.

Materials and methods

Maize caryopses (Zea mays, L. Pioneer hybrid 3906) were grown under similar conditions as described before (Cramer and Bowman, 1991a). Seedlings in a vermiculite medium were irrigated daily with 0.25 Hoagland solution and grown under constant conditions (25°C, 200 μmol m⁻² s⁻¹ PAR). One day before experiments were started, seedlings were transferred to and the roots immersed in an aerated 0.25 Hoagland solution (100 ml) in a graduated glass cylinder. The solution was shielded completely from light with aluminium foil, to prevent isomerization of ABA.

In an experiment designed to assess the timing of ABA action on leaf growth, plant roots were removed to reduce the time needed for ABA to get to the site of action (the leaf growing zone). The procedures for root removal have been published previously (Cramer and Bowman, 1991b). Root removal had no effect on leaf elongation rates for up to 6 h after root removal. In all other experiments routine assays of ABA were done with intact plants.

Mixed-ABA was purchased from Sigma (mixed isomers). Racemic ABA was purchased from Aldrich and was resolved into optically pure forms, greater than 99% pure by HPLC, according to the method described by Dunstan et al. (1992).

ABA estimation by RIA

The RIA procedures of Quarrie et al. (1988) were used for ABA analysis. This assay has been validated to be free from immunoreactive contamination for maize (Quarrie et al., 1988). The growth zone of the third leaf was used for ABA estimation. The outer leaves were removed and a 2 cm basal segment above the crown node was excised and immediately frozen in liquid nitrogen. The sections were then freeze-dried and stored over silica gel in the dark at room temperature. The freeze-dried samples were ground to a fine powder before being extracted overnight at 2–5°C with distilled, deionized water added in the ratio of 40:1 (solvent volume:leaf dry weight). Assays were carried out in triplicate for each sample.

Preliminary experiments were performed with MAC62, which is highly specific for (+)-ABA, but the supply was exhausted and MAC252 was substituted. MAC252 is a re-cloned version of MAC62 which was purchased from S Quarrie at the John Innes Centre, Colney, Norwich, NR4 7UJ, UK. Concentrations of ABA were calculated from the radioactivity in cpm present in the pellets. Standard curves with ABA were produced using serial dilutions (4000, 2000, 1000, 500, and 250 pg per vial) and logit transformation of the corrected data. The transformed data were plotted against the ln of unlabelled ABA present per vial. This RIA, using MAC252, was not found to be specific for (+)-ABA. MAC252 could not discriminate between either (+) or (−)-ABA, since standard curves were identical for (+)-ABA, (−)-ABA and mixed-ABA (Sigma mixed isomers). Values are the means of two replicates. Lines were fitted by linear regression. The concentration of mixed-ABA was divided by half to account for the lack of reaction with the (±) trans isomers (50% of total). Mixed-ABA is 25% (+) and 25% (−) cis, trans ABA. The data indicate that MAC252 reacts equally well with the (−) enantiomer of ABA as it does with the (+) enantiomer and does not react with the trans isomers.

(+)-ABA estimation by ELISA

An ELISA for (+)-ABA was purchased in kit form from Sigma (formerly sold by Idtek). The assay was performed according to the instructions in the kit. This assay was found to be highly specific for (+)-ABA over the range of ABA concentrations used in this study (data not shown). Thus, using both the ELISA and RIA, it was possible to discriminate between the natural (+)-ABA and (−)-ABA. If the RIA estimated ABA concentrations above constitutive levels, but the ELISA did not, then increased concentrations were considered to be (−)-ABA. It is considered that these assumptions are reasonable since metabolism of (−)-ABA would not be significant over the time-course of this study (Balsevich et al., 1994).

Determination of growth parameters

Growth parameters of the Lockhart equation were determined using an applied-tension technique (Cramer and Bowman, 1991a). Estimations of the growth parameters by the applied-tension technique are comparable to values derived from the pressure-block technique (Cramer and Schmidt, 1995) and the guillotine psychrometer technique (Nonami and Boyer, 1990a, b). Leaf elongation rates (LER) were measured with a displacement transducer attached to the 3rd leaf of 8-d-old maize plants.

Measurement of the length of the growing zone is necessary.
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for proper estimation of the relative elongation rate (RER) used in the above analyses. The length of the growing zone was measured by piercing the growing zone of plants treated with ABA for 4 h with a pin every 3 mm and determining the length between holes 24 h later. Water relations were determined using a pressure chamber and vapour pressure osmometer as before (Cramer and Bowman, 1991a).

Statistical analyses
All data were analysed with the MacIntosh program, SuperANOVA version 1.11, by Abacus Concepts, Inc., Berkeley, CA. All data were first analysed by ANOVA to determine significant ($P \leq 0.05$) treatment effects. Significant differences between individual means were determined using Fisher’s Protected Least Significant Difference.

Results
Effects of applied ABA on growth
Leaf elongation of intact plants was reduced by the addition of 1 μM mixed-ABA to the nutrient solution (Fig. 2). There was a lag time of about 90 min before LER declined rapidly. Treatment with 2 μM mixed-ABA caused LER to decline about 30 min earlier (data not shown). When roots were removed from plants under the surface of the solution culture (1 μM mixed-ABA), the lag time in response to ABA was shortened to less than 30 min. This indicates that a large part of the delay in growth inhibition is due to uptake and transport through the roots and that the action of ABA on elongation is fast.

In order to assess the mechanisms by which growth is inhibited by ABA, the plants must reach steady-state rates of LER, because the Lockhart equation that defines the growth parameters is based upon the assumption of steady-state conditions. Furthermore, it is important to assess this early in the response in order to minimize secondary or feed-back responses. It was found that LER declined to a lower steady-state level in about 4 h compared to its pre-ABA treatment values and remained steady for at least 24 h after the addition of mixed-ABA (Fig. 3A). ABA treatment decreases LER of wheat and barley in a similar manner (Munns, 1992). Removal of mixed-ABA from the nutrient solution resulted in a rapid recovery of LER, reaching the pre-ABA treatment rates in about 3 h. This indicates ABA effects are reversible, and that there are no permanent restrictions to growth.

Changes in (+)-ABA concentrations in the growing zone of leaves were detected within 1 h after the addition of the mixed-ABA to the nutrient solution of intact plants.

Fig. 2. Representative examples of the effects of 1 μM mixed-ABA on leaf elongation rate of the third leaf of maize plants with or without roots. At least four replications of this experiment were performed. ABA was applied 30 min after excision of roots. Growth rates were unaffected by the excision. Roots were removed under solution with a razor blade prior to treatment. The removal of roots probably permitted more rapid uptake of ABA to the growing zone.

Fig. 3. The effect of 1 μM mixed-ABA on the leaf elongation rate (mean ± SE; $n=8$) and on the (+)-ABA concentrations (mean ± SE; $n=4$) in the growing zone and mature zone of the third leaf of maize over time. (+)-ABA was estimated (not (±)-ABA because MAC62 was used in the assay. The data for Fig. 3B is from a different set of plants than set of plants used for Fig. 3A.
Fig. 4. The effect of externally applied mixed-ABA (to the root solution) on leaf elongation rate (mean ± SE; n = 8) of the third leaf of maize at 7 or 22 h after treatment.

Fig. 5. Leaf elongation rate after 22 h (same as Fig. 4) as a function of internal concentrations of (+)-ABA in the growing zone of the third leaf at 22 h (mean ± SE; n = 8). (+)-ABA was detected by RIA with MAC252.

Table 1. Comparison of (+)-ABA concentrations (mean ± SE; n = 3) in aqueous extracts of maize leaf growing zones using a RIA with MAC62 antibody highly specific for (+)-ABA.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(+)-ABA concentration (ng g⁻¹ DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>151 ± 21</td>
</tr>
<tr>
<td>1 μM mixed-ABA (4 h)</td>
<td>346 ± 21</td>
</tr>
<tr>
<td>80 mM NaCl (4 h)</td>
<td>342 ± 11</td>
</tr>
</tbody>
</table>

Therefore, it is considered that this treatment will raise (+)-ABA concentrations to a range that is experienced by salt-stressed plants.

The length of the growing zone of the third leaf was unaffected by 1 μM mixed-ABA for at least 24 h (Fig. 6). This lack of effect is important because changes in the growing zone can affect plots of LER versus turgor used for estimation of growth parameters and, therefore, must be considered when estimating growth parameters. This lack of effect on the length of the growing zone is similar to response of salt-stressed plants (Cramer, 1992; Cramer and Bowman, 1991a) and ABA-treated barley (Dodd and Davies, 1996).

Measurements of the apparent turgor indicated that there was no change in turgor in mixed-ABA treated plants after 4 h of treatment when leaf elongation reached a new but reduced steady-state rate (Table 2).

Applying a tension-force to the leaf increased LER (Fig. 7) and enabled estimation of other growth parameters (Cramer and Bowman, 1991a). The growth coefficient, mL/(mL + L), measured as the slope of the line...
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Fig. 6. The effect of 1 \(\mu\)M mixed-ABA on the length (mean \(\pm\) SE; \(n=8\)) of different segments along the growing zone of the third leaf. The growing zone was punctured with a needle every 3 mm above the leaf base (root–shoot junction) and the space between the holes was measured 24 h later. An increase in distance above 3 mm represents cell expansion in the segment (delineated by the holes). In the lower two segments, holes were obscure or had completely disappeared in control plants and were only detected in a few plants treated with mixed-ABA.

in Fig. 7A, was unaffected by mixed-ABA treatment, and therefore not the cause of reduced \(LER\) by \((+)-ABA\) (Table 2).

Plastic and elastic extension (Fig. 7B, C) also were unaffected by \((+)-ABA\), indicating \(m\), the cell wall extensibility, was not affected (Table 2). Both \(m\) and \(mL/(m+L)\) were unaffected by \((+)-ABA\), indicating that \(L\), the hydraulic conductance, was also not affected (Table 2).

The apparent yield threshold, \(Y\), is generally defined as the minimum pressure required for cell expansion, and is thought to be a property of the cell wall. \(Y\) is estimated as the \(x\)-intercept of plots of \(RER\) compared with the growth force. Note that the elongation force is the sum of the turgor and the applied elongation force. Once \(LER\) reached steady-state (4 h after treatment), \(Y\) was increased above that of controls by \((+)-ABA\), thus decreasing the effective turgor for growth (Table 2). All

![Graph](image)

Table 2. The effect of \((+)-ABA\) and \(NaCl\) on cell growth parameters (mean \(\pm\) SE; \(n=10\)) of the 3rd leaf of maize (8-d-old plants)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>ABA</th>
<th>Control(^a)</th>
<th>ABA(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turgor (MPa(^{-1}))</td>
<td>0.48 (\pm) 0.02</td>
<td>0.49 (\pm) 0.02</td>
<td>0.48 (\pm) 0.02</td>
<td>0.48 (\pm) 0.01</td>
</tr>
<tr>
<td>(m) (min(^{-1}) MPa(^{-1}))</td>
<td>1.93 (\pm) 0.2 (\times) 10(^{-2})</td>
<td>2.0 (\pm) 0.11 (\times) 10(^{-2})</td>
<td>1.79 (\times) 10(^{-2})</td>
<td>1.9 (\times) 10(^{-2})</td>
</tr>
<tr>
<td>(L) (min(^{-1}) MPa(^{-1}))</td>
<td>5.37 (\times) 10(^{-3})</td>
<td>5.32 (\times) 10(^{-3})</td>
<td>5.52 (\times) 10(^{-3})</td>
<td>5.52 (\times) 10(^{-3})</td>
</tr>
<tr>
<td>(mL/(m+L)) (min(^{-1}) MPa(^{-1}))</td>
<td>4.2 (\pm) 0.37 (\times) 10(^{-3})</td>
<td>4.2 (\pm) 0.29 (\times) 10(^{-3})</td>
<td>4.1 (\times) 10(^{-3})</td>
<td>4.1 (\times) 10(^{-3})</td>
</tr>
<tr>
<td>(Y) (MPa)</td>
<td>0.13 (\pm) 0.04</td>
<td>0.18 (\pm) 0.03</td>
<td>0.13 (\pm) 0.01</td>
<td>0.18 (\pm) 0.02</td>
</tr>
</tbody>
</table>

\(^a\)From Cramer (1992).
of these responses are remarkably similar to the effects of salinity on growth (Table 2).

**Differential LER response to ABA enantiomers**

Mixed-ABA (Sigma mixed isomers) was used initially, because it was the cheapest form of ABA. Mixed-ABA consists of 50% ($\pm$) cis,trans and 50% ($\pm$) trans,trans isomers. The trans,trans isomer is not known to have any effect on growth, whereas the (+) or (−) enantiomer of the cis,trans isomer can be variable (Walton, 1983). In order to ascertain which isomers were affecting growth, optically pure isomers were added independently to the nutrient solution (Fig. 8). The effects on growth are presented for treatments after 22 h; similar results were found for all treatments after 7 h. LER was affected by the cis, trans isomers, but not the trans, trans isomers (the racemic mixture was not significantly different from mixed-ABA). LER was very sensitive to (+)-ABA, because LER was reduced by external concentrations as low as 200 nM (+)-ABA. External concentrations of 100 nM had no effect on LER (data not shown). LER was less sensitive to external concentrations of (−)-ABA, because LER was only reduced significantly at external concentrations above 2 µM. However, LER was equally sensitive to internal concentrations of either (+) or (−)-ABA (Fig. 9). (−)-ABA was detected by RIA using MAC252, which was equally sensitive to (+)- and (−)-ABA enantiomers (Fig. 1). This was verified by ELISA (Sigma, Idetek) in which ABA-binding to the antibody in this assay was unaffected by the (−)-enantiomer (data not shown but see Walker-Simmons et al., 1991). It would appear the lower sensitivity to external (−)-ABA concentrations is due to the lower ability of the growing zone to absorb the (−)-ABA compared to the (+)-ABA (Fig. 10) (Balsevich et al., 1994; Windsor et al., 1994, 1992), and not to higher rates of metabolism, because (−)-ABA is metabolized at a slower rate than (+)-ABA in maize cells (Balsevich et al., 1994).

![Fig. 8. The effect of (+)-ABA, (−)-ABA, racemic ABA and mixed-isomers ABA on leaf elongation rate (mean ± SE; n=8) at 22 h after treatment. ABA was added to the solution culture. The concentration of mixed-ABA was divided by half to account for the lack of reaction with the (±) trans, trans isomers (50% of total).](image)

![Fig. 9. Leaf elongation data from Fig. 8 at 22 h plotted against its internal ABA concentration (mean ± SE; n=8) of the third leaf at 22 h. Inhibitor concentration was detected by RIA with MAC252 and verified by ELISA.](image)

![Fig. 10. The effect of external concentration of (+)-ABA and (−)-ABA on their respective internal concentrations in the growing zone of the third leaf of maize (mean ± SE; n=8).](image)
Discussion

Application of (+)-ABA to the nutrient solution inhibited LER of maize. The inhibition was rapid, occurring within 30 min of the addition of (+)-ABA. Furthermore, LER fully recovered upon removal of external mixed-ABA, coinciding with the disappearance of (+)-ABA in the growing zone, whereas (+)-ABA concentrations in mature tissue were unaffected. In addition, the mixed-ABA concentration in the solution was not necessarily a good predictor of effect. Thus, it is important to correlate ABA effects with internal ABA concentrations in the growing zone, not mature leaf concentrations or external concentrations.

Plants were treated with mixed-ABA for 1 d before removal of ABA from the external solution. This is plenty of time for both genetic and structural adjustments to occur. The fact that inhibition of LER was fully reversible indicates that (+)-ABA did not have permanent limitations on LER. Thus, structural features such as thicker cell walls, which would restrict cell elongation, are not likely to be significant factors affecting leaf cell expansion.

(+)-ABA, at natural internal concentrations, only affected the apparent yield threshold of the cell wall. This is consistent with the reversible effects of (+)-ABA. The apparent yield threshold can rapidly change (up or down) and is a property that is dependent upon metabolism (Green et al., 1971). Furthermore, other growth hormones are known significantly to affect the apparent yield threshold of the cell wall (Behringer et al., 1990; Maruyama and Boyer, 1994; Okamoto et al., 1990). ABA is known to stimulate or inhibit the hydraulic conductivity of roots (Collins and Kerrigan, 1974; Ludewig et al., 1988; van Steveninck et al., 1988), but this did not account for the growth inhibition in this study. It has also been reported that ABA inhibits cell wall extensibility in maize coleoptiles (Kutschera and Schopfer, 1986a, b), but the plants were treated with 100–250 μM ABA (racemic mixture), concentrations that would produce much higher internal ABA concentrations than that found in control or naturally-stressed plants. Thus, this effect on cell wall extensibility at this concentration of external ABA is probably unnatural for maize.

Steady-state LER was inversely proportional, in a hyperbolic manner, to steady-state (+)-ABA concentrations in the growing zone. A similar response was found when leaf expansion was plotted against internal leaf ABA concentrations of two Brassica species (He and Cramer, 1996) and internal growing zone ABA concentrations of two Gramineae species (Dodd and Davies, 1996). This indicates that LER is highly correlated to internal ABA concentrations of the growing zone, at least at steady-state conditions. However, ABA concentrations were poorly correlated with LER prior to steady-state conditions (Fig. 3), which may mean that ABA distribution in the tissue was different than that at steady-state.

Although internal growing zone ABA concentrations were found to be highly correlated with leaf elongation of ABA-treated plants, this correlation was not very good for drought-stressed plants (Dodd and Davies, 1996). In that study there appeared to be an interaction between drought and ABA indicating an increase in sensitivity of the drought-stressed plants to ABA. No such interaction or change in sensitivity was found in salt-stressed Brassica species (He and Cramer, 1996).

Most studies of the effects of ABA use a racemic mixture, probably due to the high cost of optically pure (+)-ABA. It is clear that the optical isomers of ABA can have different physiological (Walton, 1983) and genetic activity (Dong et al., 1994; Wilen et al., 1993). Thus, studies using racemic mixtures of ABA may be misleading. From the observation that optical isomers of ABA differ in their uptake and metabolism, it has been suggested that only (+)-ABA be used for ABA uptake, transport and accumulation studies (Windsor et al., 1992).

There are very few reports which compare the effects of (+)- and (−)-ABA on leaf elongation (Walton, 1983). In this study, the effect of the (+)-enantiomer was equal to the (−)-enantiomer, when endogenous concentrations were compared. The (−)-enantiomer is less effective exogenously, because it accumulates in the growing zone to a lesser extent.

In conclusion, the inhibition of LER by applied ABA is best correlated to the internal concentrations of (+)-ABA. The inhibition of LER by ABA is caused by an increased apparent yield threshold of the cell wall. The (+)-ABA accumulates to a much higher concentration than (−)-ABA. Thus, at low external concentrations (<2 μM), the effect of a racemic or mixed-ABA treatment is primarily determined by the (+)-ABA concentration.

At higher external racemic or mixed-ABA concentrations, the (−)-enantiomer has significant effects on LER. The usefulness of measuring internal ABA concentrations is that it enables us to rationalize the dose-response of (+)- and (−)-ABA, to more accurately detect the speed of the response, and it will allow for comparisons with other species, analyses (i.e. sensitivity analyses) or other assay systems. The similarity of leaf growth in response to ABA and salinity indicates that ABA is a plausible candidate for regulating LER in salt-stressed plants. In a forthcoming paper the hypothesis that ABA causes the growth inhibition of salt-stressed plants will be specifically addressed.

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References


