Auxin-Induced Growth of Avena Coleoptiles Involves Two
Mechanisms with Different pH Optima

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ABSTRACT

Although rapid auxin-induced growth of coleoptile sections can persist for at least 18 hours, acid-induced growth lasts for a much shorter period of time. Three theories have been proposed to explain this difference in persistence. To distinguish between these theories, the pH dependence for auxin-induced growth of oat (Avena sativa L.) coleoptiles has been determined early and late in the elongation process. Coleoptile sections from which the outer epidermis was removed to facilitate buffer entry were incubated, with or without 10 micromolar indoleacetic acid, in 20 millimolar buffers at pH 4.5 to 7.0 to maintain a fixed wall pH. During the first 1 to 2 hours after addition of auxin, elongation occurs by acid-induced extension (i.e. the pH optimum is <5 and the elongation varies inversely with the solution pH). Auxin causes no additional elongation because the buffers prevent further changes in wall pH. After 60 to 90 minutes, a second mechanism of auxin-induced growth, whose pH optimum is 5.5 to 6.0, predominates. It is proposed that rapid growth responses to changes in auxin concentration are mediated by auxin-induced changes in wall pH, whereas the prolonged, steady-state growth rate is controlled by a second, auxin-mediated process whose pH optimum is less acidic.

The acid-growth theory (6, 19) states that auxin causes receptive cells to excrete protons, and that the resulting acidification of the apoplastic solution permits cell loosening and, thus, cell elongation to occur. This theory is supported by the ability of acidic solutions to induce elongation of stem and coleoptile tissues as rapid as that induced by auxin (4, 17). However, although AIG of coleoptiles can persist at a rapid rate for at least 18 h, the acid-induced elongation persists for only a few hours (10, 13, 17).

Three theories have been proposed to explain the difference in duration of these two responses (Fig. 1). The first (Fig. 1A) states that wall loosening at all times during AIG occurs by the same mechanism that requires an acidic apoplast. Auxin keeps the apoplast acidic by promoting H+ excretion from the cells, but in addition, it has a second role of maintaining the ability of the walls to be loosened by acid (2, 19). In the absence of auxin, acidic solutions cause wall loosening and, thus, elongation. However, the capacity for further acid-induced wall loosening is used up in the process, the walls lose their ability to undergo additional wall loosening, and acid-induced extension ceases. The second theory (Fig. 1B) states that the initial phase of auxin-induced elongation is due to acid-induced wall loosening, but that this is superceded by a second, auxin-mediated growth mechanism that does not require the wall to be as acidic (24, 25) and involves a different mechanism of wall loosening. The third theory (Fig. 1C) states that auxin-induced wall loosening at all times is due to a mechanism whose pH optimum is >5, and that acid-induced extension does not occur in vivo (10, 11, 15, 23).

Existing evidence did not enable us to distinguish between these three theories (see 'Discussion'). One way to do so is to determine the pH optima for elongation during both the early (0–2 h) and later (2–12 h) stages of auxin-induced growth, and to determine whether auxin can promote elongation when the wall pH is fixed by strong pH buffers. If theory A is correct, the pH optimum will be identical and <5 in both stages. During the first phase, auxin will cause no additional elongation in well-buffered tissues because no auxin-induced change in wall pH can occur under these conditions, whereas in the second phase, auxin will promote elongation because it is regenerating the capacity of the walls to be loosened by acid. With theory B, the pH optimum will also be <5 in the early stage, but the optimum will be >5 in the later stages. With C, the pH optimum will be above that needed for acid-induced wall loosening at all times, and auxin will promote growth in pH-buffered tissues during both early and late phases, because it is causing wall loosening by some mechanism that does not require a change in wall pH. This investigation was undertaken to determine the pH dependency of AIG of oat (Avena sativa L.) coleoptile sections at both early and later stages of growth. Because such pH optima can be obtained only if all cells are accessible to external pH buffers, the outer epidermis was first removed from the sections. The wall pH was maintained with strong buffers and the pH dependence of elongation was determined.

MATERIALS AND METHODS

Seedlings of oat (Avena sativa L., cv Victory) were grown in moist vermiculite for 4 d at 25°C under dim red light (<1 \( \mu \text{mol} \text{ m}^{-2} \text{s}^{-1} \)). Coleoptiles 25 to 32 mm long were selected, and unless otherwise stated, the epidermis was removed (peeled) as two strips with jewelers' forceps. This removes 80 to 100% of the outer epidermis, as measured by uptake of neutral red into the peeled areas. Groups of 5 or 10 5-mm

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2 Abbreviations: AIG, auxin-induced growth (growth with auxin minus growth without auxin); FC, fuscoscin; KS, medium containing 5 mM KCl and 10 mM sucrose.
Early Phase  Later Phase
0–2 h  2–12 h

A.  X → H+ → WL  X → H+ → WL
    Auxin  Auxin  Auxin

B.  X → H+ → WL  Y → WL
    Auxin  Auxin

C.  Y → WL  Y → WL
    Auxin  Auxin

Figure 1. Three theories to explain the difference in persistence of acid-induced and AIG. In A, acid-induced wall loosening (WL) occurs in both early and later phases, whereas auxin is also needed to resupply some factor (X) needed as a substrate for acid-induced wall loosening. In B, the early phase involves acid-induced wall loosening, but in the later phase, an auxin-induced increase of a different factor (Y) leads to a different type of wall loosening. In C, both early and later phases are due to nonacidic wall loosening, although acid-induced wall loosening can occur if the walls become sufficiently acidic.

sections were cut, starting 3 mm from the tip, measured with a microscope fitted with an eyepiece micrometer, and placed immediately into Petri dishes containing 10 mL of solution. Unless otherwise stated, the solution contained 5 mM KCl + 10 mM sucrose (+KS) and 20 mM Mes-NaOH buffer, pH 4.5 to 7.0, to maintain a constant pH in the apoplast. The pH of the apoplastic solution will not necessarily be identical to that of a buffered external solution, even if the buffer can penetrate into the walls. This is indicated by the fact that a 20 mM Mes-NaOH buffer at pH 6.5 failed to inhibit FC-induced elongation completely (data not shown). It is not feasible to use buffer concentrations in excess of 20 mM because of osmotic inhibition of growth (data not shown). The concentration of 20 mM for the buffer was chosen as the best compromise between maximum buffer effectiveness and minimum osmotic inhibition of growth. It must be remembered, therefore, that pH values given are for solution pH and not cell wall pH, although these should be closely correlated. IAA was used at 10 μM because this gives an optimal growth response with both intact and peeled Avena coleoptile sections. The dishes were incubated on a rotary shaker (50 rpm) at 25°C under dim red light. The sections were remeasured at intervals as indicated in individual figures. AIG was calculated at each time as the elongation in the presence of IAA minus the elongation in the absence of IAA.

A number of variations in procedure were tried without any measurable change in the results. These include changing the solution after each measurement, the use of K-phosphate buffers or Mes-KOH buffers instead of Mes-NaOH, and the incubation of the sections in 5 mL of solution in 25-mm diameter test tubes on a Rollardrum (1 rpm). In some experiments, the pH of solutions was measured after the incubation; in the pH range from 5.5 to 7.0 there was <0.1 pH unit change in the solution during incubation, whereas in the pH range from 4.0 to 5.5, the solution pH often increased by up to 0.2 pH units over 12 h.

All experiments reported here were repeated at least five times. Data presented are for representative experiments. Values in the figures are ± se (n = 5 or 10).

RESULTS

To ensure the accessibility of the external buffer to the apoplast of all cells, the epidermis with its cuticular barrier was first removed (peeled) from the sections. Because it has been claimed that such sections cannot undergo normal AIG (10, 11), it was first necessary to demonstrate that the loss of the outer epidermis does not seriously alter the AIG response. When sections with or without the outer epidermis were incubated in 20 mM Mes-NaOH, pH 6.5, + KS (the conditions used in most of the remainder of these experiments), both sets of sections elongated rapidly and with nearly identical kinetics in response to auxin (Fig. 2). The total extension under these conditions was reduced as compared with solutions with low buffer, where extensions of over 100% in 20 h were obtained for both intact and peeled sections (3).

The time course for AIG of peeled Avena coleoptile sections in the presence of 20 mM Mes-NaOH + KS showed two distinct phases (Fig. 3). In the first hour, the extension varied inversely with the solution pH. Thereafter, the growth rate changed to a new, constant rate that was maintained for the remainder of the 12 h. During this second phase, the growth rate at pH 5.5 exceeded that at pH 4.5 or 6.5.

Figure 2. Comparison of the growth response of intact (UP) versus peeled (P) Avena coleoptile sections. Sections were incubated in 20 mM Mes-NaOH, pH 6.5, 5 mM KCl, and 10 mM sucrose, with (+) or without (−) 10 μM IAA.
The pH profile for growth of peeled sections during the first hour, with and without auxin, is shown in more detail in Figure 4. At pHs 6.5 and 6.0, the extension rate was uniformly low. A decrease in pH to 5.5 caused a large increase in rate, with further increases occurring as the pH was lowered to 4.5. The solution at pH 5.0, the apoplastic pH believed to exist in auxin-treated coleoptiles (1, 9), caused elongation greater than that induced by auxin in unpeeled sections in 1 h. Auxin caused no increase in rate at pHs between 4.5 and 6.5 during this first hour. This is expected

Figure 3. Time course of extension of peeled Avena coleoptile sections in response to IAA and in the presence of 20 mM Mes-NaOH buffers at pH 4.5, 5.5, and 6.5. Sections were incubated with 5 mM KCl, 10 mM sucrose, and 20 mM Mes-NaOH at the indicated pH with (+) or without (−) 10 μM IAA.

Figure 5. Difference in lag time before the onset of AIG in water versus pH 5 or 6 buffer. Sections were incubated with 5 mM KCl and 10 mM sucrose with or without 10 μM IAA and with or without 20 mM Mes-NaOH at either pH 5.0 or 6.0. AIG is the extension with IAA minus the extension without IAA at each time point. AIG in water is greater than in pH 5 or 6 buffer because of the lower osmotic concentration in the solution.

Figure 4. Effect of buffer pH and IAA (Aux) on the growth of peeled Avena coleoptile sections during the early period (0–1 h). Sections were incubated for 1 h with 5 mM KCl, 10 mM sucrose, and 20 mM Mes-NaOH at the indicated pH. Bars show the growth of unpeeled (UP) sections with (+) or without (−) 10 μM IAA during the first hour.

Figure 6. pH profile for auxin-induced and control growth during the later phase (1–12 h) of growth of peeled Avena coleoptile sections. Sections were incubated in 5 mM KCl and 10 mM sucrose with (+) or without (−) 10 μM IAA and with 20 mM Mes-NaOH at pH 4.5 to 6.5. Extension for the period from 1 to 12 h after addition of auxin was determined.
Extrapolation of the conducted variances from the onset of the experiment eliminates the possibility that absorbable auxin is involved in the acid-induced extension. This is because the removal of the epidermis might have rendered the sections unable to respond to auxin for the first 1 to 2 h after addition of auxin. This possibility has been shown to be incorrect by the fact that when the sections were incubated without any buffer so that auxin-induced wall acidification could still occur, the AIG commenced after a 20- to 30-min lag (Fig. 5).

The pH profiles for the growth of auxin-treated sections in KS (Fig. 6) and for the auxin-mediated component of the growth (Fig. 7) during the period between 1 and 12 h showed a pH optimum of 5.5 to 6.5. The presence of buffers at either higher or lower pHs resulted in a decrease in growth rate. This is also seen in Figure 3. Similar pH profiles were obtained for the growth measured between 2 and 3 or 12 and 13 h after addition of auxin (data not shown). These data indicate that AIG during this period is controlled by some process different from the one that controls the growth rate during the early period. If KCl and sucrose were omitted from the medium, the total amount of AIG was reduced due to a lack of absorbable solutes (22). However, the curve for AIG showed an even sharper optimum at pH 6.0, and there was only a small amount of AIG at either pH 4.5 or 6.5 (Fig. 7).

Because incubation of peeled sections in a pH 6.5 buffer eliminates the first acid-extension phase, the timing of the onset of the second auxin mechanism could be determined. Extrapolation of the AIG curves back to zero growth indicated that the lag prior to the onset of the second growth phase was about 90 min (Fig. 8). In seven experiments, the lag varied from 65 to 115 min, with most close to 90 min. Similar experiments conducted in the presence of more acidic buffers showed a similar 90-min lag prior to the onset of AIG (Fig. 5).

During the first phase of AIG, the capacity of the walls to be loosened by acidic solutions is used up during the elongation (2). This does not delay the onset of the second phase, which suggests that the set of load-bearing bonds cleaved during the second phase may be different from those involved in the acid-induced extension. But, can the walls respond to acid after the onset of the second phase, or has the elongation during the second phase eliminated the capacity for acid-induced wall loosening? To test this, sections were incubated with auxin at pH 6.0 to allow the second auxin-growth phase, but not the acid extension, to take place. After 5 h, some sections were transferred from pH 6.0 to 4.5 (Fig. 9). There was still a distinct growth response to the acidic solution, but the magnitude of the response was considerably reduced as compared with sections incubated from the first at pH 4.5. This suggests that there is some overlap in the load-bearing bonds involved in the two auxin-growth processes.

**Figure 7.** pH profile for AIG of peeled Avena coleoptile sections during the second phase of growth. Sections were incubated in 20 mM Mes-NaOH buffers, pH 4.5 to 6.5, without KCl or sucrose and with or without 10 μM IAA (∅) and the AIG determined between 1.5 and 11.5 h after addition of auxin, or incubated in 5 mM KCl and 10 mM sucrose with or without 10 μM IAA (●) and the AIG determined between 1 and 12 h.

**DISCUSSION**

The marked difference in duration of acid extension versus AIG has long been noted (10, 16, 19, 26), and constitutes a serious problem for the acid-growth theory. Three theories have been proposed to explain this discrepancy (Fig. 1), but the evidence to support any one theory is limited.

Vanderhoof and colleagues have presented three observations from their studies with soybean hypocotyl sections to support theory B that the early and later phases of AIG have different mechanisms and that only the early phase involves...
Figure 9. Demonstration that peeled *Avena* coleoptile sections can undergo acid-extension after the onset of the second growth mechanism. Sections were incubated with 5 mM KCl, 10 mM sucrose, 10 μM IAA, and 20 mM Mes-NaOH at pH 4.5 or 6.0. At 5 h (arrow), one set of sections was transferred from pH 6.0 to 4.5 buffer.

acid extension. The first observation is that cytokinins inhibited the later phase of AIG, but not the initial phase (25). The second observation is that a 1 mM HEPES pH 7.0 buffer inhibited the initial phase, but not the later phase of AIG (24). On the other hand, a 50 mM K-phosphate pH 6.0 buffer failed to inhibit either phase of the growth of their intact sections (24), whereas Rayle and Cleland (20) reported that pH 6.5 buffers were equally effective in inhibiting both early and later phases of AIG in abraded soybean hypocotyl sections. Finally, they observed that the rate of AIG rose to a maximum, then fell again before rising to a second maximum; the two maxima were taken as evidence that two auxin mechanisms must be operating (24, 26). Pearce and Penny (14), however, explained the two maxima as being a consequence of differences in the rate at which different cell layers respond to auxin. In coleoptiles, the presence of two distinct maxima have sometimes been seen (13), but in other cases, only a single maximum followed by a plateau was observed (13, 21). These observations suggest that different mechanisms are responsible for controlling the initial and later phases of AIG, but they do not indicate that the later phase is acid insensitive. The available data are equally consistent with theory A, in which the second phase requires a second auxin-mediated step prior to acid-induced wall loosening.

The evidence to support theory A comes primarily from studies on the capacity of *Avena* coleoptile cell walls to undergo *in vitro* acid-induced extension. This capacity is reduced by prior treatment of sections with acid, either given exogenously or produced endogenously in response to FC (2), and is enhanced, on the other hand, by auxin (2). Although there are striking parallels between the *in vitro* acid-extension and *in vivo* AIG (18), doubts about the correlation between the two exist (16).

The third theory (C), which states that no part of AIG is due to acid-induced wall loosening, is primarily supported by reports that the apoplastic pH of auxin-treated coleoptiles, which is believed to be about 5.0 (1, 9), is not low enough to account for the rate of auxin-induced elongation (10, 11, 21); if the extension is not acid-mediated, it must be mediated by a completely different mechanism. Recently, evidence was presented (4) indicating that for abraded coleoptile and stem tissues, the auxin-induced acidification is sufficient to explain the initial growth response to auxin, and the data presented here indicate the same for peeled *Avena* coleoptiles. Theory C does not, in fact, specify a particular pH optimum for AIG, but does state that in buffered solutions, auxin will promote growth equally in early and late phases because a change in apoplastic pH is not required in this mechanism. The fact that in buffered solutions AIG does not occur during the first 90 min, but does occur thereafter (Fig. 8), is inconsistent with this theory.

The data presented here indicate that the early and later phases of growth of auxin-treated *Avena* coleoptile tissues have different pH optima. The initial expansion is dependent on the acidity of the apoplast, with an optimum pH <5.0. Because the growth rate during this period is strictly dependent on the apoplastic pH, it is difficult to demonstrate any promotion of growth by auxin in buffered solutions, but a strong and early auxin effect (Fig. 5) can be detected if unbuffered solutions are used. Kutschera et al. (11) claimed that the initial extension of peeled coleoptiles is simply a release of tension (i.e., an elastic swelling). The fact that this extension is inhibited by neutral buffers (Fig. 5) and by cold and by metabolic inhibitors (data not shown) indicates that it occurs by a biochemical wall-loosening mechanism instead. The data for this early period of AIG are equally consistent with theories A and B.

The extension during the later phase of AIG, on the other hand, has a pH optimum of 5.5 to 6.0 (Fig. 7). In fact, AIG is inhibited at pH 4.5 relative to 5.5. Could the reduced growth rate at pH 4.5 be due to acid-induced damage to the cells? Although acidic solutions can be toxic to coleoptile cells (7), the constancy of the growth rate of auxin-treated sections at pH 4.5 between 1 and 12 h (Fig. 3) speaks against this. In addition, toxic effects have been observed only when the pH is 4.0 or below (7). If the solution does not contain KCl and sucrose, the inhibition at pH 4.5 and 6.5 is greatly enhanced (Fig. 7). This may mean that the buffers are more effective in maintaining the wall pH in the absence of these solutes. It may be a coincidence that this pH optimum is the same as the optimum for binding of auxin to the 22-kD auxin-binding protein of maize coleoptiles (12).

The mechanism of wall loosening during this second phase is not known. It may involve a completely different set of load-bearing bonds (theory B). On the other hand, the actual wall-loosening process may be the same in both phases and may be optimal at pHs below 5 (theory A). During the first phase, the pH dependence of the wall-loosening process would predominate because it is using preexisting substrate (i.e., sets of load-bearing bonds). But during the second phase, the rate-limiting step would presumably be the generation of new load-bearing bonds for the wall-loosening enzymes. If the wall loosening during this second phase involves cleavage...
of the same sets of bonds (theory A), the rate of generation of new bonds (X in Fig. 1) must be less than 25% as great at pH 5.0 as at pH 6.0, and 10% as great at pH 4.5. Although the data are not incompatible with theory A, they suggest that theory B is more likely.

The initiation of the second phase of AIG occurs with a lag of about 90 min (Fig. 8). Auxin-induced gene activation may be required for this phase. Although the SAUR genes are rapidly activated by auxin (28), there is no evidence that the resulting proteins are produced within the first hour, and they may not reach the wall for 90 min. The possibility that AIG requires wall synthesis has been suggested repeatedly (16). This wall synthesis may constitute an essential part of the second auxin-growth mechanism. This idea is supported by the fact that galactose, which is believed to act by inhibiting wall synthesis, effectively inhibits only this later phase of growth (27). It would also be consistent with the fact that in vitro cell walls, which do not have the capacity for wall synthesis, can undergo acid extension, but not prolonged extension at pH 6 (5).

These data might seem to indicate that acid-induced extension makes only a small contribution to the total AIG. For example, assuming an apoplastic pH of 4.8, acid-mediated extension may be too small to contribute to the total elongation (Figs. 4 and 6). However, the magnitude and persistence of acid-induced extension may be greatly underestimated from these experiments because removal of the epidermis may have caused sufficient damage to limit this type of extension. In support of this idea is the fact that incubating peeled coleoptile sections without auxin for 1 h markedly reduces their ability to undergo acid-mediated extension (4).

Under steady-state conditions, the growth rate is presumably determined by the second growth mechanism. Changes in auxin concentration would cause a slow readjustment of the growth rate if there is also a 90-min lag between the change in concentration and the change in the growth rate. But in two cases, gravitropic and phototropic curvature, rapid responses occur to local changes in the auxin concentration (8). These responses may be too rapid to be caused by the second growth mechanism. It seems more likely that such rapid changes in growth are mediated by the acid-growth mechanism. It has been shown (Fig. 9) that the walls are still able to respond to changes in pH even after the second growth mechanism is well underway.

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LITERATURE CITED