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Relationship between RNA- and Protein-Synthesis and Cell Wall Acidification in Auxin-Mediated Elongation of Sunflower Hypocotyls

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해바라기 하배축의 오옥신 유도 신장에서 RNA 및 단백질의 합성과 세포벽 산성화의 관계

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ABSTRACT

The roles of RNA- and protein-synthesis and H^+ excretion in IAA (10 μM)-induced elongation were investigated using abraded hypocotyl segments of sunflower (*Helianthus annuus* L.). The response of elongation initiated about 13 min after IAA treatment. Removal of cuticle, acting as diffusion barrier for inhibitors, by mechanical abrasion of hypocotyl segments enhanced the effect of inhibitors markedly, but the degree of abrasion for the saturated effect of inhibition was different among inhibitors. The elongation induced by IAA was completely inhibited when cycloheximide (10 μM) was applied to abraded hypocotyl segments as shortly as 4 min before the onset of the growth response (=10 min after administration of IAA). Cordycepin (200 μM) prevented completely IAA-induced elongation when applied as shortly as 19 min before the onset of the growth response (=5 min before administration of IAA). Vanadate (1 mM) inhibited both IAA-induced elongation and medium acidification via IAA-induced H^+ excretion to apoplast. Cycloheximide and cordycepin also prevented IAA-induced H^+ excretion strongly. However, inhibition by cycloheximide of IAA-induced elongation was not alleviated by acidifying the cell wall to pH 4.5. The results indicate that, a few minutes before the initiation of growth, protein synthesis is demanded for the initiation of IAA-induced elongation and the H^+ excretion to cell wall, and that the H^+ excretion, even though it may be necessary for elongation, does not seem to bring about acid growth simply through acidifying cell wall.

INTRODUCTION

The significance of protein- and RNA-synthesis and H^+ excretion to cell wall in the initiation of auxin-mediated cell elongation has been debated for several decades to be still unsolved. In the 1960s, the gene-activation hypothesis suggested that auxin regulates the synthesis of specific mRNA coding for proteins (growth-limiting proteins, GLP) necessary for the growth process (Key, 1969). This hypothesis was supported by observations that in long-term (several hours) auxin increased the absolute amounts of RNAs and proteins (Noodén and Thimann, 1963; Trewavas, 1968; Key, 1969; Verma *et al.*, 1975; Zurfluh and Guilfoyle, 1982). However, because short-term (up to 1 h of exposure) auxin effects on gene expression were not detected even though auxin fully stimulated growth within 15 to 25 min (Evans and Ray, 1969; Jacobs and Ray, 1976), the attention was away from gene expression. Alternatively, the acid-growth theory, which proposed that cell elongation is initiated by auxin-induced

proton excretion (Rayle and Cleland, 1970; Hager *et al.*, 1971), was advanced.

In the short-term kinetics of auxin-induced cell elongation, the inability of early studies to measure directly the change in amounts of proteins and RNAs was due to the limit of techniques available at that time (Trewavas, 1976), and the results that protein synthesis inhibitor like cycloheximide (CHI) could not inhibit auxin-induced elongation were caused by the non-removed epidermal cuticle which acts as diffusion barrier for the inhibitor (Bates and Cleland, 1979; Edelmann and Schopfer, 1989). Using technique of molecular biology, recently, early auxin-mediated mRNA changes were founded with lag period of less than 10-15 min (Guilfoyle, 1986; Theologis, 1986) even though the functions of their products have not been known. Moreover, the evidence from inhibitor experiments with abraded tissues indicated that very short-lived proteins were necessary for auxin-mediated elongation (Bates and Cleland, 1979; Edelmann and Schopfer, 1989). With all these evidences for 'neo-gene-activation hypothsis', it is evident that auxin-induced H^+ excretion occurs in both coleoptiles and dicotyledonous stems and, when this process is blocked by plasma membrane H^+-ATPase inhibitor vanadate, auxin-mediated elongation is inhibited (Theologis, 1987; Brummell and Hall, 1987). The evidences that protein- and RNA-synthesis inhibitors also inhibited the auxin-induced H^+ excretion and elongation (Bates and Cleland, 1979 and 1980; Rayle and Cleland, 1980; Theologis *et al.*, 1985) indicate that auxin-induced cell wall acidification through H^+ excretion is preceded by protein and RNA synthesis. From these evidences, Bates and Cleland (1980) suggested, in accordance with acid-growth theory, that GLP comprises a part of the H^+ excretion mechanism and Theologis (1986) proposed that protein products by the primary auxin event are related to the secretory pathway of cell wall materials, but Edelmann and Schopfer (1989) claimed that the independence of CHI and COR (cordycepin) inhibition of pH made it rather unlikely that GLP is directly involved in the mechanism of auxin-mediated H^+ excretion.

The aim of the present work was to provide further information concerning this uncertainty. Therefore, we examined the effect of the cuticular barrier against other inhibitors (COR and vanadate) as well as CHI in order to investigate the exact timing of action of these inhibitors, and the effect of these inhibitors on auxin-mediated elongation and cell wall acidification.

MATERIALS AND METHODS

Growth and preparation of plant material. Sunflower (*Helianthus annuus* L.) seeds were surface-sterilized in 1% sodium hypochlorite solution for 30 min. After thorough rinsing, they were soaked for 20 h in distiled water with aeration, then grown on vermiculite moistened with distiled water in the dark at 25℃ for 4 days. Seedlings with the elongating hypocotyl between 5 and 6 cm in length were selected and a 10 mm segment excised from 2-3 mm below the apical hook. Hypocotyls were abraded before cutting the segments by pulling the hypocotyls ten times through fined sea-sand powder (5 to 100 μm-mostly 10 μm-in diameter with irregular shape) between thumb and forefinger. Hypocotyls treated in this way showed a regular pattern of neutral red (5 g/*l*) stained patches but were virtually unstained by Evans Blue. Above twenty times abrasion, however, blue patches started to appear. Since neutral red is accumulated in living cells whereas Evans Blue stains only damaged cells (Taylor and West, 1980), this test indicates that the abrasion procedure applied effectively disrupts the cuticle without injuring the underlying epidermal cells (Schopfer, 1989). The hypocotyl segments were incubated on distiled water for 2 h before use in order to deplet endogeneous active auxins. All experiments were done at normal room light.

Chemicals. Cycloheximide and sodium orthovanadate (Na_3VO_4) were purchased from Sigma, cordycepin from Boehringer and indole 3-acetic acid from Merck.

Measurement of growth. Growth kinetics were measured by either one of two procedures: 1) For measurement of long-term growth, ten segments were floated on 10 m*l* medium in a glass test tube (25 mm ϕ). A stream of air was bubled through the medium. Up to 12 units of this type were placed in a thermostated water bath (25± 0.2℃). Segment elongation was read at suitable intervals by eye on a millimeter scale. 2) For measurement short-term kinetics, single segments were placed in a linear-displacement transducer (Serie 605-2.5, Erichsen, Wuppertal, FRG) which had a sensitivity of 1.68 mV/μm and permitted the measurement of segment elongation rates with an accuracy of ± 1 μm/min (Kutschera and Schopfer, 1985a). The voltage output of the transducer was fed into a digital meter displaying both the actual voltage and the voltage change per unit time (set to 1 min).

Transducer voltage output (i.e., segment extension) and

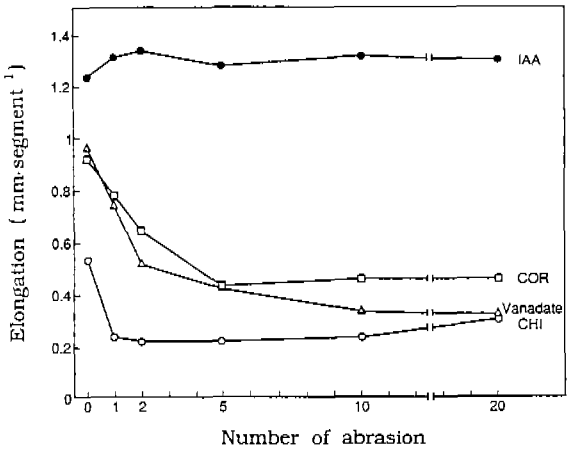

Fig. 1. Growth of sunflower hypocotyl segments according to the number of abrasion in only IAA (10 μM) and some elongation inhibitors (COR, 200 μM; Vanadate, 1 mM; CHI, 10 μM) in 10 μM IAA. The hypocotyl segments were abraded 0 to 20 times with fine sea sand powder (See materials and methods). Elongation was determined 2 h after the treatment.

voltage increment per min (i.e., segment extension rate) were recorded with a two channel chart recorder set to 6 mm/h.

Measurement of pH. One millimolar potassium phosphate buffer was prepared at pH 7.0 and hypocotyl segments were placed into this buffer with aeration, in which the tissue-to-volume ratio used were 30 segments/3.0 m*l*. The pH of the solution was measured at suitable intervals with a pH meter (Fisher Accumet Model 230A pH/ion meter).

Data points represent means of two to six independent measurements. Short-term growth kinetics are presented as growth-recorder tracings from single representative experiments.

RESULTS AND DISCUSSION

Effect of cuticle abrasion on the permeability of inhibitors. The epidermal cuticle of hypocotyl segments acted as diffusion barrier for inhibitors used in this experiment (Fig. 1). In contrast to these inhibitors, the effectiveness of IAA was not significantly affected by the degree of cuticle abrasion, therefore the cuticle does not seem to inhibit the entry of IAA. In short-term kinetics of IAA-induced elongation of maize (Kutschera and Schopfer, 1985a) and oat (Schopfer, 1989) coleoptiles, abrasion has no significant effect but caused decrease of elongation by 30% in long-term. Moreover, in azuki bean (Branca *et al.*, 1988), IAA-induced elongation was about 55% less in abraded than in intact segments even though the percent growth amount due to the addition of the hormone was about the same in intact and abraded segments.

In this work, the effectiveness of inhibitors on inhibition of IAA-induced elongation was different with the degree of cuticle abrasion. For protein synthesis inhibitor cycloheximide (CHI, 10 μM), in nonabraded segments the inhibitor showed saturating effect at 50 μM, however even with single time abrasion by fine sea sand powder (diameter in c.a. 10 μm), the effectiveness of the inhibitor was increased by more than 60%. In nonabraded segments, RNA synthesis inhibitor cordycepin (COR, 200 μM) and plasma membrane H^+-ATPase inhibitor vanadate (1 mM) inhibited the IAA-induced elongation by 20% but when the segments were abraded 10 times the inhibitory effect of these inhibitors was increased to 65% and 70%, respectively.

According to our results, each inhibitors differently demanded the degree of abrasion for a saturating effect of inhibition on the IAA-induced elongation. Besides these inhibitors, ion such as Ca^{2-} also can not practically penetrate the cuticle barrier (unpresented data). Therefor, in order to examine the correct action concentration and the most effectiveness of inhibitors (or chemicals), the cuticle barrier of stem must be removed.

Effect of cuticle abrasion on the action of CHI. The effect of cuticle abrasion on the permeability of inhibitors could been shown more obviously by investigating the effect on the action of CHI. The inhibitory effect of 50 μM CHI in nonabraded segments was the same as that of 10 μM in abraded segments (Fig. 2). In this experiment, we used 10 μM CHI, above which a saturating effect of the inhibition on IAA-induced elongation was shown.

In nonabraded segments, this concentration of CHI could not prevent IAA-induced elongation even when applied at the same time as, or within 1h before the hormone, although the elongation rate was considerably reduced with longer CHI pretreatments (Fig. 3). On the other hand, in abraded segments the rate of IAA-induced elongation was dropped as shortly as 5 min after administration of 10 μM CHI (Fig. 4b and c). The results of Fig. 3 would lead to the false conclusion that CHI is incapable of preventing IAA-induced growth. In early experiments (Barkley and Evans, 1970; Penny, 1971; Mura-

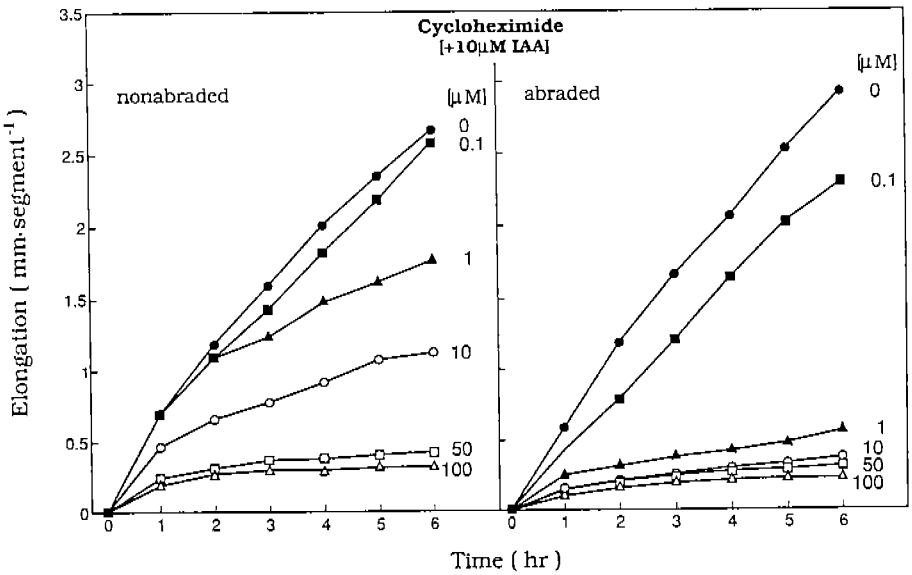

Fig. 2. Effect of CHI on IAA-mediated elongation of nonabraded (left) and abraded (right) sunflower hypocotyl segments. IAA (10 μM) and CHI (0-100 μM) were added simultaneously at time zero (=2 h after cutting the segments).

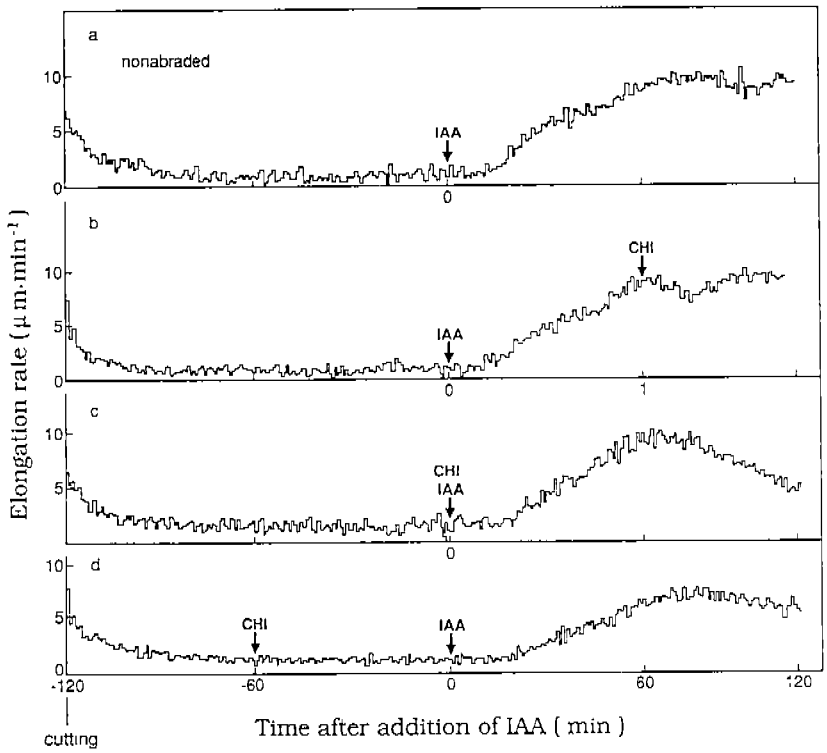

Fig. 3. Effect of CHI (10 μM) on the short-term elongation kinetics of nonabraded sunflower hypocotyl segments. IAA (10 μM) was added at time zero (=2 h after cutting the segments); CHI was added 1 h after (b), simultaneously with (c), and 1 h before (d) IAA. These data were presented as growth-recorder tracings from single representitive experiments.

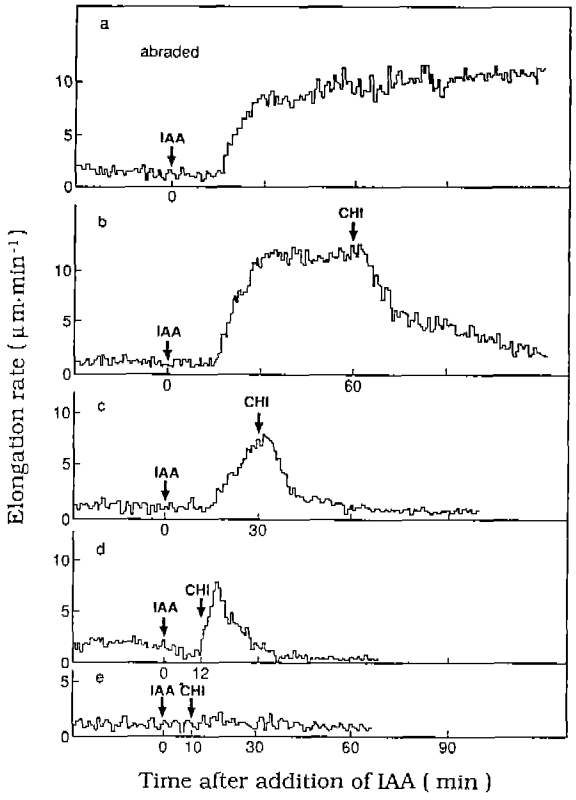

Fig. 4. Effect of CHI (10 μM) on the short-term elongation kinetics of abraded sunflower hypocotyl segments. IAA (10 μM) was added 2 h after cutting the segments (=time zero); CHI was added 60 min (b), 30 min (c), 12 min (d), and 10 min (e) after IAA.

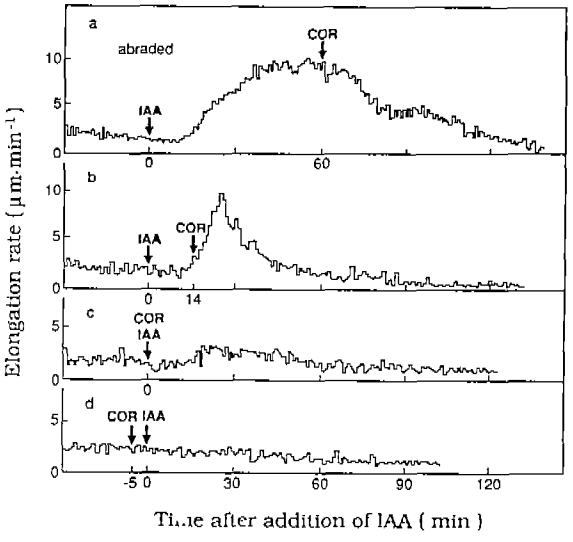

Fig. 5. Effect of COR on IAA-mediated elongation of abraded sunflower hypocotyl segments. IAA (10 μM) and COR (0-200 μM) were added simultaneously at time zero (=2 h after cutting the segments).

yama and Ueda, 1973; Vanderhoef *et al.*, 1976), in which cuticle was not considered as a diffusion barrier, also CHI could not prevent short-term IAA-induced elongation. From the results that CHI had no significant effect on the early elongation event by auxin in lupin and soybean hypocotyls repectively, Penny (1971) and Vanderhoef *et al.* (1976) proposed that the inhibition of auxin-induced elongation is dependent on GLP pool being alredy on existence and GLP synthesis at the level of protein synthesis is necessary for the late elongation phase. These early studies made the misinterpretation that protein synthesis is not demanded for the initiation of auxin-induced elongation. Problems of these studies, however, were pointed out by Bates and Cleland (1979). As Edelmann and Schopfer (1989) emphasized more clearly about the cuticle abrasion, in this context, our results provide further information that one should effectively remove cuticle for the exact inhibitor study about auxin-induced stem elongation.

Effect of CHI on IAA-induced elongation in abraded hypocotyl segments. In sunflower hypocotyl segments, IAA-induced elongation started 13.3± 1.5 min after administration of the hormone (Figs. 3a and 4a), and the elongation rate reached the maximum value 30 min after IAA then continued for several hours. When CHI was added after attaining a steady-state elongation rate, in abraded segments the elongation rate dropped rapidly with 5 min lag time and consequently dropped to zero 60 min after applying the inhibitor (Fig. 4b). Even when CHI was added at the initiation of maximum rate (i.e. 30 min after IAA administration), the elongation rate rapidly reduced within 5 min and it was inhibited completely 30 min after adding CHI (Fig. 4c). The complete inhibition of the initiation of IAA-dependent growth was accomplished when CHI was added 10 min after IAA treatment (i.e. 3-5 min before the onset of growth response). These results indicate that the synthesis of protein (s) with very short life span (less than 5 min) is necessary for initiation and maintenance of IAA-induced growth. In similar investigation done with abraded maize coleoptiles (Edelmann and Schopfer, 1989), also it was suggested that auxin-dependent growth needs for protein (s) having life time as short as 5 min. However, no pro-

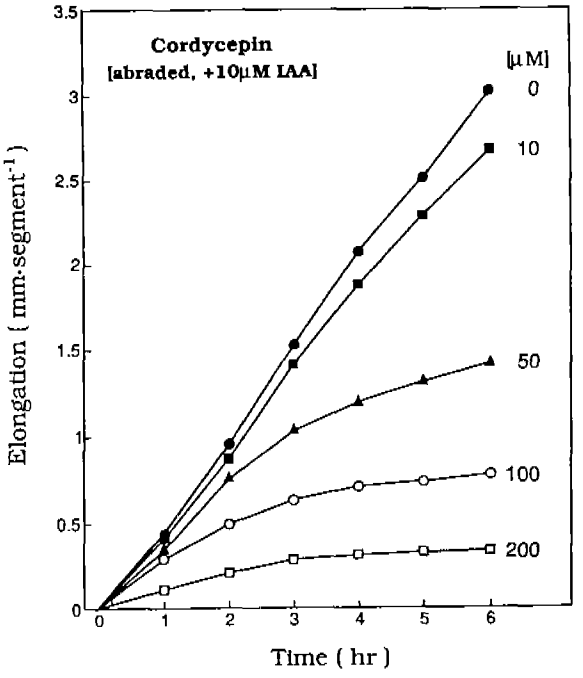

Fig. 6. Effect of COR (200 μM) on the short-term elongation kinetics of abraded sunflower hypocotyl segments. IAA (10 μM) was added 2 h after cutting the segments (=time zero); COR was added 60 min after (a), 14 min after (b), simultaneously with (c) and 5 min before (d) IAA.

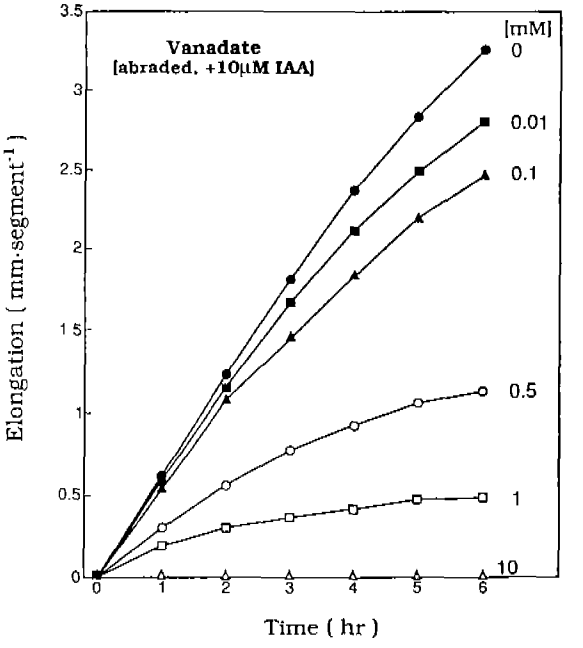

Fig. 7. Effect of vanadate on IAA-mediated elongation of abraded sunflower hypocotyl segments. IAA (10 μM) and vanadate (0-10 mM) were added simultaneously at time zero (=2 h after cutting the segments).

teins with such extremely short life time have been identified and moreover no their functions have been clearly known.

With these uncertainty, it has been proposed that GLP may be hydrolytic enzyme for glucan degradation (Sakurai and Masuda, 1979), GLP comprises a part of H^+ excretion mechanism (Bates and Cleland, 1980), or GLP may be a small pool of structural protein(s) incorporated into the epidermal cell wall in order to maintain its plastic extensibility necessary for growth (Edelmann *et al.*, 1989).

Effect of COR on IAA-induced elongation in abraded hypocotyl segments. In long term, RNA synthesis inhibitor cordycepin (COR) at concentration of 200 μM showed nearly complete inhibitory effect on the IAA-dependent growth in abraded sunflower hypocotyl segments (Fig. 5). Thus we used this concentration for the study of short-term elongation kinetics and medium acidification.

Similarly to CHI, Fig. 6 shows the effect of COR on the short-term kinetics of IAA-induced elongation. 200 μM COR began to inhibit the steady-state elongation rate with about 10 min lag time and completely inhibited the elongation rate 70 min after administration of the inhibitor (Fig. 6a). When COR was added simultaneously with IAA, the initiation of IAA-induced elongation was slightly shown (Fig. 6c). The complete inhibitory effect of COR appeared when COR was added 18 min before the initiation of IAA-induced growth (i.e. 5 min before administration of IAA). COR, similarly to CHI, inhibited the IAA-induced elongation rate more rapidly in sunflower hypocotyl (in this experiment) than in maize coleoptiles (Edelmann and Schopfer, 1989). In Avena coleoptiles (Sakurai and Masuda, 1979), the inhibitory effect of COR (100 μM) on the auxin-dependent growth and glucan degradation was more rapid than that of sunflower hypocotyls in this work. These evidences suggest that the synthesis of mRNA coding GLP is necessary for the initiation of auxin-mediated growth. The timing of the inhibitory kinetics of COR in our result was consistent with the reports that the level of auxin-dependent mRNA increased within 5-10 min after auxin administration (Hagen and Guilfoyle, 1985; Theologis *et al.*, 1985).

Effect of vanadate on IAA-induced elongation on abra-

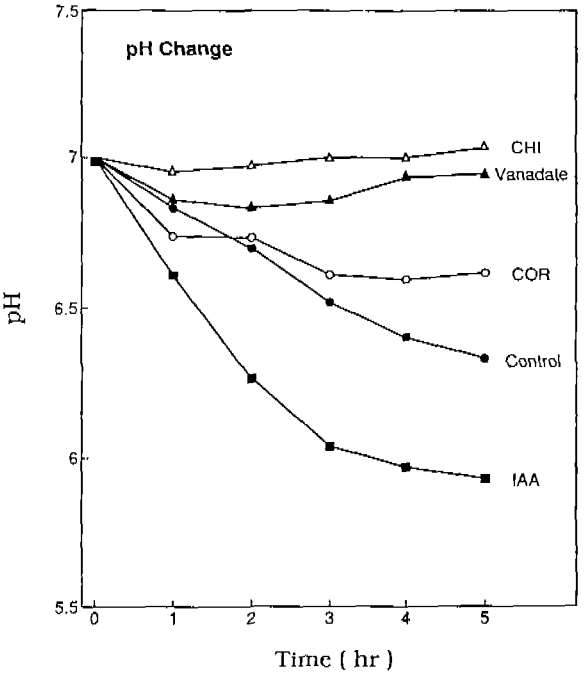

Fig. 8. Effect of some elongation inhibitors on acidification of the 3 ml medium by 30 abraded sunflower hypocotyl segments. The segments were incubated at time zero (=2 h after cutting the segments) in 10 μM CHI, 1 mM vanadate, 200 μM COR, 10 μM IAA or control (1 mM potassium phosphate buffer, pH 7.0). Each medium included 1 mM potassium phosphate buffer (pH 7.0) and the inhibitor media were added with 10 μM IAA.

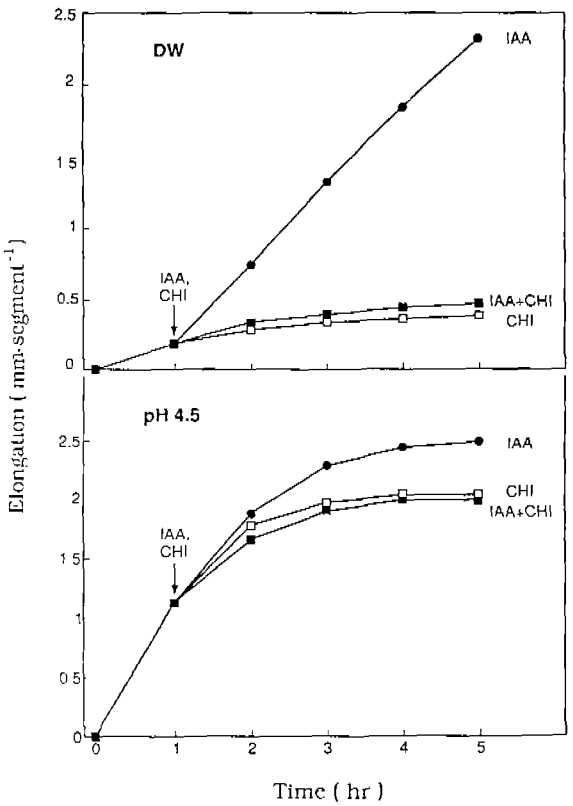

Fig. 9. Effect of CHI on IAA-mediated elongation of abraded sunflower hypocotyl segments in acidic buffer (5 mM citrate buffer, pH 4.5). IAA (10 μM) and CHI (10 μM) were added 1 h after time zero (=1 h after cutting the segments) as indicated by the arrow.

ded hypocotyl segments. In accordance with acid-growth theory, it was proposed that, in responsive cells, auxin stimulates growth by activating a plasma membrane-bound ATPase which transfer protons from the cytosol into the cell wall space (Hager *et al.*, 1971). In this point of view, the inhibition of auxin-induced elongation and cell wall acidification by vanadate (plasma membrane H^+-ATPase inhibitor) has been used as the evidence for acid-growth theory. However, there has been suggestion that the inhibitory effect by vanadate on auxin actions should be reinterpretated (Brummer *et al.*, 1984; Brummell, 1986).

Vanadate, in sunflower hypocotyl segments, began to inhibit considerably IAA-induced elongation at the concentration of 0.5 mM and then 1 mM vanadate showed a similar inhibitory level to 10 μM CHI and 200 μM COR (Fig. 7). This concentration was used for the experiment about medium acidification. In cucumber hypocotyls (Brummell, 1986), pea epicotyls and oat coleoptiles (Jacobs and Taiz, 1980), 1 mM vanadate showed nearly complete inhibitory effect on auxin-dependent growth.

Inhibitory effect of vanadate on auxin-dependent growth signifies the requirement of H^+ excretion by plasma membrane ATPase in auxin-induced growth, however this result not necessarily leads us to a conclusion that cell wall acidification, the consequence of H^+ excretion to wall, is necessary for the growth (Brummell, 1986).

Effect of inhibitors on IAA-induced medium acidification in abraded hypocotyl segments. Ten μM IAA acidified the media including abraded segments from pH 7.0 to pH 5.9 after 5 h (Fig. 8). pH of control medium also fell by 0.6, but less than 1.1 in IAA medium. Prevention of medium acidification by inhibitors for auxin-indu-

ced growth was shown in Fig. 8, in which 10 μM CHI and 1 mM vanadate completely and 200 μM COR considerably inhibited the acidification. CHI inhibited the auxin-induced acidification in maize coleoptiles (Kutschera and Schopfer, 1985b) and sunflower hypocotyls (Mentze *et al.*, 1977), and vanadate inhibited the acidification in cucumber hypocotyls (Brummell, 1986) and pea epicotyls and oat coleoptiles (Jacobs and Taiz, 1977). Our result that the IAA-induced acidification was blocked by COR means that IAA-induced H^+ excretion may be controled at the level of RNA synthesis. This is consistent with the result that α-amanitin (5 μM) inhibited IAA-induced cell wall acidification as well as elongation in pea epicotyls (Theologis *et al.*, 1985).

Evidences that inhibitors for auxin-induced growth prevent auxin-induced proton excretion have been used for acid-growth theory (Evans, 1985), however, this must be differently interpreted according to the following present result and the result from maize (Edelmann and Schopfer, 1989).

Effect of CHI on IAA-induced elongation in acid solution. In accordance with acid-growth theory, the fact that CHI inhibits both auxin-induced H^+ excretion and elongation dues to the inhibition of synthesis of protein(s) related to cell wall aicidification. If this explanation were true, auxin-induced growth inhibited by CHI would be recovered by infiltering cell wall with acid solution. However, as shown in Fig. 9, this could not be proved. Coleoptiles and dicotyledonous stem usually show acid-mediated growth in buffer with pH below 5.0 (Brummell and Hall, 1987). pH 4.5 citrate buffer (5 mM) induced rapid elongation of sunflower hypocotyl segments within 1 h and this buffer caused acid growth even in CHI-treated segments. However, IAA-mediated growth inhibited by CHI was not restored to the level of IAA-induced growth and remained at the same level of only CHI treatment. This data supports the result from maize coleoptiles (Edelmann and Schopfer, 1989).

CONCLUSIONS

At many studies related with auxin-induced stem elongation, false concentration and timing at which growth inhibitors act have been shown. Our results, in this context, indicate that the cuticle abrasion test for each inhibitor (or other chemicals) should be preceded.

This investigation, through inhibitor study with abraded sunflower stem segments, suggests that short-lived proteins (or RNAs) are necessary for auxin-mediated elogation (Figs. 4 and 6) and also that auxin-induced H^+ excretion needs protein (and RNA) synthesis (Fig. 8). In addition, the growth inhibition by vanadate (Fig. 7) indicates that H^+ excretion is demanded for auxin-mediated elongation. However, the growth inhibition by CHI do not likely occur through the inhibition of cell wall acidification (Fig. 9). Although this investigation do not present the physiological function of the protien(s)whose synthesis is inhibited by CHI or induced by auxin, the results from Figs. 8 and 9 mean that the protein(s) could be related to the H^+ excretion mechanism but H^+ excretion itself do not cause acid growth through wall acidification.

ACKNOWLEDGEMENT

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적 요

큐티클층이 제거된 해바라기 (*Helianthus annuus* L.) 하배축 절편의 오옥신 (IAA, 10 μM) 유도 신장 반응에 있어서 RNA, 단백질 합성 및 세포벽으로의 H^+ 방출과의 관계에 대해 조사하였다. 하배축의 표피조직 바깥에 있는 큐티클층은 실험에 사용하는 여러가지 대사억제제에 대해 어느 정도 불투과성 장벽으로 작용하여 억제제의 효과를 저하시키지만, 곱게 간 석영사로 하배축 절편을 문질러서 큐티클층을 제거하였을 때 억제제들의 효과는 현저하게 증가하였다. 단백질 합성 억제제인 cycloheximide (CHI, 10 μM)는 5분 정도의 지연시간 뒤에 IAA유도 신장반응을 억제하기 시작하였으며, 신장 반응 4-5분 전 (IAA처리 10분 뒤)에 처리하였을 때 IAA에 의한 신장반응을 완벽하게 억제하였다. 그러나 신장률이 정상상태를 유지하고 있는 IAA처리 60분 뒤에 CHI를 처리하였을 때는 신장률이 0에 이르기까지 60분 이상이 걸렸다. RNA합성 억제제인 cordycepin (COR, 200 μM)은 IAA보다 5분 먼저 처리하였을 때 신장반응을 완전히 억제하였으며, 정상상태의 신장을 완전히 억제하는 데는 70분 이상이 걸렸다. 원형질막 H^+-ATPase의 활성을 저해하는 vanadate (1 mM)는 IAA유도 신장과 세포벽으로의 H^+ 방출을 통한 배양액의 산성화를 모두 억제하였다. 또한 CHI는 완벽하게, COR 역시 현저하게 IAA에 의한 H^+ 방출을 억제하였다. 그러나 산성용액속에서, CHI에 의한 IAA 유도 신장의 억제가 다시 회복되지 않는 것으로 보아 CHI가 단순히 세포벽의 산성화를 억제하여 신장을 저지하는 것같지는 않다. 이상의 결과에서

해바라기 하배축의 IAA유도 신장 반응의 시작과 세포벽으로의 H^+ 방출에는 단백질(생장제한 단백질)의 합성이 필요하며 이 단백질은 신장이 시작하기 전에는 존재하지 않고 IAA에 의한 신장 반응 몇 분전에 새로 합성됨을 추측할 수 있다. 그리고 COR이 IAA 유도 신장을 억제한다는 것은 IAA에 의한 생장제한 단백질의 합성이 RNA합성 수준에서 이루어 진다는 것을 의미한다. 또한 신장 반응에는 세포벽으로의 H^+ 방출이 필요하나 이는 단순히 세포벽의 산성화를 통한 산성생장의 원인이 되는 것 같지는 않다.

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