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Hydroxyl radical-induced cell-wall loosening *in vitro* and *in vivo*: implications for the control of elongation growth

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Summary

Hydroxyl radicals (OH) are capable of unspecifically cleaving cell-wall polysaccharides in a site-specific reaction. I investigated the hypothesis that cell-wall loosening underlying the elongation growth of plant organs is controlled by apoplastically produced OH attacking load-bearing cell-wall matrix polymers. Isolated cell walls (operationally, frozen/thawed, abraded segments from coleoptiles or hypocotyls, respectively) from maize, cucumber, soybean, sunflower or Scots pine seedlings were pre-loaded with catalytic Cu or Fe ions and then incubated in a mixture of ascorbate + H_2O_2 for generating OH in the walls. This treatment induced irreversible wall extension (creep) in walls stretched in an extensiometer. The reaction could be promoted by acid pH and inhibited by several OH scavengers. Generation of OH by the same reaction in living coleoptile or hypocotyl segments caused elongation growth. Auxin-induced elongation growth of maize coleoptiles could be inhibited by OH scavengers. Auxin promoted the production of superoxide radicals (O_2^-), an OH precursor, in the growth-controlling outer epidermis of maize coleoptiles. It is concluded that OH fulfils basic criteria for a wall-loosening factor acting in auxin-mediated elongation growth of plant species with widely differing cell-wall polysaccharide compositions.

Keywords: cell-wall loosening, coleoptile growth, elongation growth, hydroxyl radicals, hypocotyl growth, *Zea mays*.

Introduction

Cell extension growth in turgid plant organs is brought about by chemically loosening the structure of growth-limiting cell walls, resulting in the relaxation of wall tension and concomitant osmotic water uptake. The biochemical mechanism of this wall-loosening reaction has not yet been elucidated, although numerous potential wall-loosening proteins have been investigated (Cosgrove, 1999; Cosgrove, 2000; Hoson, 1993). These proteins have the capacity to either catalyse the enzymatic (hydrolytic) degradation of particular cell-wall polysaccharides *in vitro*, or belong to the family of expansins that cause stretch-dependent extension (creep) in acidified cell walls of killed tissues by breaking intermolecular non-covalent bonds (Cosgrove, 1999; Cosgrove, 2000; Fry, 1995). Creep-inducing expansins are thought to mediate the growth responses that can be induced by cell-wall acidification with acid buffers or fusicoccin (Cosgrove, 1998). In agreement with the proposed mode of expansin action in the cell wall, 'acid growth' induced by these experimental treatments is often not accompanied by a decline in hemicellulose (xyloglucan) amount or molecular weight, as found in corresponding experiments with auxin (Sakurai *et al.*, 1977; Talbott and Ray, 1992). In contrast to expansin-mediated 'acid growth', the growth responses induced by auxin appear to be generally correlated with a degradation of cell-wall polysaccharides. This important distinction between the wall-loosening processes underlying acid-induced and hormone-induced growth is supported by numerous lines of experimental evidence. For instance, elongation growth induced by auxin in pea seedling internodes is accompanied by an increase in soluble polysaccharides in the apoplastic fluid, resulting from the breakdown of hemicellulosic polymers, presumably xyloglucans (Labavitch and Ray, 1974; Terry *et al.*, 1981). Using interference microscopy, Bret-Harte *et al.* (1991) showed that auxin induces a breakdown of wall material in the growth-limiting outer epidermal wall of pea internode segments elongating in the absence of wall

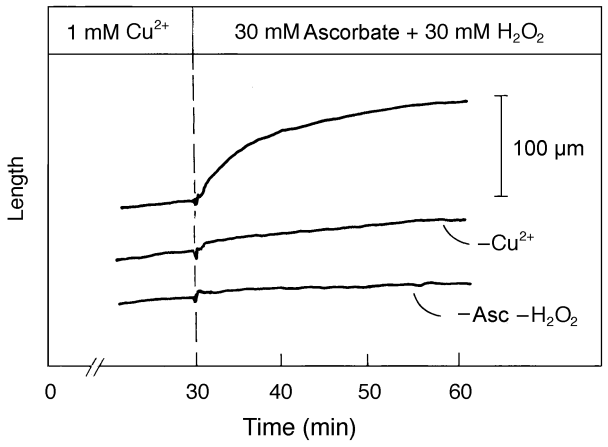

Figure 1. Induction of extension (creep) by generating OH in isolated cell walls of maize coleoptile segments.
Freshly harvested, abraded 1 cm segments, killed by freezing and thawing, were subjected to a load of 40 g in the extensiometer and pre-incubated in a solution containing 1 mM $CuCl_2$ in 50 mM sodium succinate buffer (pH 5.5). After 30 min the medium was replaced by 30 mM sodium ascorbate + 30 mM H_2O_2 in the same buffer. Controls were performed by omitting either $CuCl_2$ or ascorbate/H_2O_2 from the pre-incubation or post-incubation medium, respectively.

synthesis. A closer examination of the changes in polymer composition of the outer epidermal wall revealed a dramatic auxin-dependent decrease in the amount of hemicelluloses, while the cellulose remained unaffected (Bret-Harte and Talbott, 1993). Hemicellulose degradation during auxin-induced growth, demonstrated as a decrease in molecular weight of extracted material, was found in oat coleoptiles, azuki bean epicotyls, pine hypocotyls, squash hypocotyls and pea stem internodes (Talbott and Ray, 1992 and references cited therein). Although these findings point strongly to the involvement of backbone cleavage of hemicellulosic wall polymers in auxin-mediated wall loosening, the extensive research into the enzymatic base of this process *in vivo* has so far been unsuccessful. Of all the putative wall-loosening enzymes tested to date, none promotes the extensibility of isolated walls as expansins do; however, expansins loosen the wall without detectable hydrolysis of wall polymers (Cosgrove, 1998).

In this situation it could be helpful to consider alternative biochemical wall-loosening mechanisms, for instance the non-enzymatic cleavage of wall polymers by the hydroxyl radical (OH). This extremely reactive, short-lived intermediate of O_2 reduction is principally capable of splitting covalent bonds in all kinds of organic molecules in a diffusion-limited reaction, i.e. within a few nanometres of its site of formation (Halliwell and Gutteridge, 1989). OH produced by the reduction of H_2O_2 with Fe^{2+} ions (Fenton's reagent) has been shown to decompose cell-wall materials such as straw and sawdust (Halliwell, 1965). Extending preliminary experiments by Miller (1986), Fry (1998) showed that cell-wall polysaccharides such as pectin and xyloglucan can be broken down *in vitro* by OH generated in a Fenton-type reaction, e.g. by the reduction of O_2 with ascorbate in the presence of Cu ions. This finding led Fry (1998) to suggest that OH, produced in the apoplastic space of plant tissues, could act as a site-specific oxidant targeted to play a useful physiological role in cell-wall loosening processes underlying cell expansion, fruit ripening and organ abscission. Independently, similar ideas have been put forward based on the finding that plant peroxidase can catalyse the production of OH from O_2 in the presence of a suitable reductant such as NADH (Chen and Schopfer, 1999). Plant cells are potentially able to generate OH in the apoplastic space, although this has so far been shown only in germinating seeds (Schopfer *et al.*, 2001) and in response to pathogen attack (Kuchitsu *et al.*, 1995; v. Tiedemann, 1997). Biological production of OH is believed to be mediated by the reduction of hydrogen peroxide with superoxide (Haber–Weiss reaction) catalysed by Fe or Cu ions (Halliwell and Gutteridge, 1989):

$$H_2O_2 + O_2^- \xrightarrow{Fe^{2+}/Fe^{3+}\ Cu^{+}/Cu^{2+}} OH + OH^- + O_2$$

It has recently been shown that this reaction can also be catalysed by peroxidases (Chen and Schopfer, 1999) and that cell-wall polysaccharides can be depolymerized by peroxidases acting in the OH-generating mode *in vitro* (Schweikert *et al.*, 2000). Hence, as peroxidases are generally present in cell walls of growing tissues in great abundance, OH can potentially be generated there whenever H_2O_2 and O_2^- are available. As peroxidases are generally ionically or covalently bound to cell-wall polymers, OH could be generated specifically at sites where it causes effective polymer cleavage without damaging other cell constituents. This scenario is met in elongating maize coleoptiles that have been shown to produce O_2^- and its dismutation product H_2O_2 in the presence of peroxidase in the apoplastic space of the growth-controlling outer epidermis (Frahry and Schopfer, 2001). Thus the question arises whether apoplastically generated OH can indeed cause wall loosening leading to elongation growth. This question was investigated by experimentally generating OH in the cell wall and determining its effect on wall extensibility *in vitro* and *in vivo* in a selection of plant species.

Results

A convenient experimental method for chemically generating OH is based on the reduction of H_2O_2 with ascorbate in the presence of catalytic amounts of Cu or Fe ions (Halliwell and Gutteridge, 1989). The creep test (Hohl and

Schopfer, 1992; Kutschera and Schopfer, 1986) was used for investigating the effect of OH produced by this reaction on cell-wall extensibility in maize coleoptiles. In this test a segment of a killed organ ('isolated cell walls') is stretched by a constant force in an extensiometer, which allows measurement of the kinetics of induced changes in length (creep) of the wall specimen by means of a linear displacement transducer. Initial attempts to induce creep by incubating isolated cell walls in solutions containing H_2O_2, ascorbate and Cu or Fe ions were unsuccessful because the OH generated in the bathing medium did not reach the target sites in the walls during their limited lifetime. This problem was circumvented by pre-impregnating the cell walls with the metal catalyst before initiating OH formation with an ascorbate/H_2O_2 mixture. Cu and Fe ions can be bound to polysaccharides (Halliwell and Gutteridge, 1989), and this targets the generation of OH to sites in the wall where polymer cleavage can take place. Figure 1 shows that, under these conditions, OH can indeed be shown to induce maize coleoptile walls to extend. The initially high creep rate slows down continuously, presumably because of the decreasing rate of OH production due to substrate exhaustion. If the incubation medium is replaced by fresh medium after 30 min, creep can be boosted a second time (Figure 2).

In experiments designed to further characterize OH-induced cell wall extension, the following results were obtained.

1 Succinate buffers (5–100 mM succinate) were suitable for stabilizing the pH in the incubation medium without affecting the response to OH. In citrate buffers, significantly higher concentrations of Cu^{2+} were needed to obtain comparable creep rates, presumably because of the metal-chelating activity of citrate (Halliwell and Gutteridge, 1989).

2 Fe ions were as effective as Cu ions in catalysing cell-wall extension. Both metals promote creep in a comparable manner if applied in the range 0.1–10 mM during a 30 min pre-incubation period (Figure 3, inset).

3 After pre-incubation with 1 mM Cu^{2+} or Fe^{2+}, creep was increased by ascorbate + H_2O_2 in a concentration-dependent manner in the range 1–30 mM (Figure 3).

4 The pH dependence of creep was slightly different for Cu and Fe (Figure 4). Neutral pH was inhibitory compared to the moderately acidic (pH 5–6) typical for plant cell walls. Creep was strongly promoted in the lower pH range, especially if Fe was used as a catalyst. Moreover, Figure 4 shows that OH can be significantly more effective than H^+ in inducing cell-wall extension even at an unphysiologically low pH of 4.

5 OH-mediated creep produces a plastic rather than a viscoelastic deformation of the cell wall, which can be demonstrated as an irreversible increase in length after removal of the stretching force (Figure 5). This is a necessary condition for a wall-loosening reaction involved in growth (Hohl and Schopfer, 1992). The increase in segment length measured in the relaxed state after unloading exceeded the extension due to the creep reaction before unloading about threefold.

The causal involvement of OH in cell-wall extension induced by ascorbate/H_2O_2 in the presence of Cu or Fe ions was demonstrated by inhibiting the reaction with substances acting as OH scavengers (Halliwell and Gutteridge, 1989). Figure 6 shows the attenuation of the Cu-catalysed creep response by adenine, histidine or thiourea. Urea, possessing no appreciable scavenger activity for OH, did

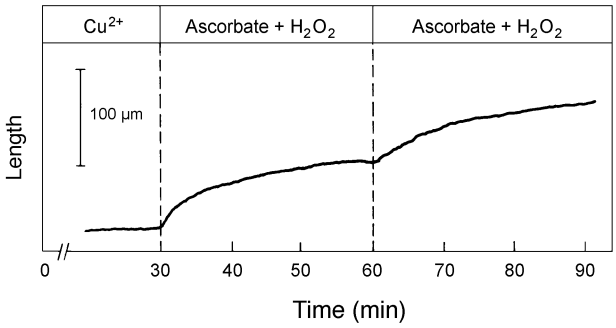

Figure 2. Re-induction of extension by re-establishing OH generation with fresh substrates in isolated cell walls of maize coleoptile segments.
Experimental conditions as in Figure 1, except that the incubation medium was replaced by fresh ascorbate/H_2O_2 medium after 60 min.

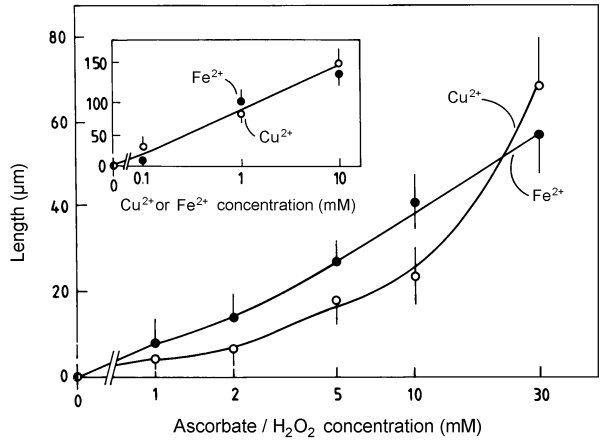

Figure 3. Dependence of extension on concentrations of ascorbate/H_2O_2 and Cu^{2+} or Fe^{2+} used for generating OH in isolated cell walls of maize coleoptiles.
Experimental conditions as in Figure 1, except that the (equimolar) concentrations of ascorbate and H_2O_2 during post-incubation were varied as indicated on the abscissa (pre-incubation: 1 mM $CuCl_2$ or $FeSO_4$, pH 6.0). Inset: effect of $CuCl_2$ or $FeSO_4$ concentration during pre-incubation (post-incubation: 30 mM ascorbate + 30 mM H_2O_2, pH 5.5). Extension was measured as the increase in segment length during 30 min post-incubation in ascorbate/H_2O_2. Extension in buffer controls was subtracted.

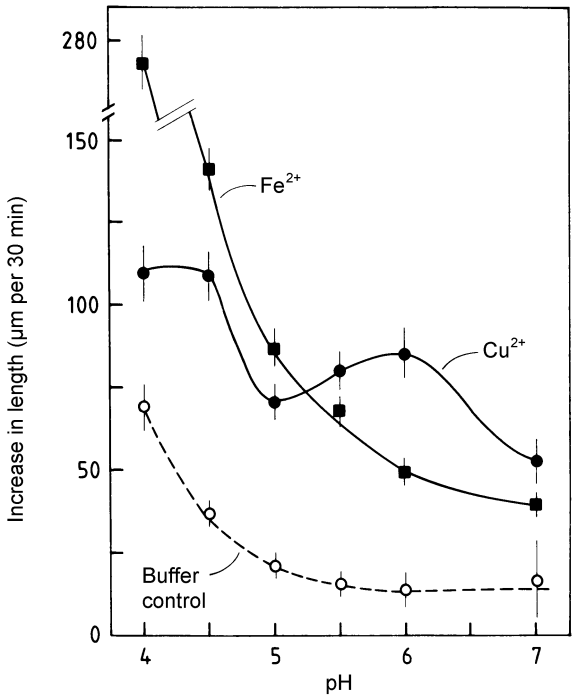

Figure 4. Dependence on pH of extension in isolated cell walls of maize coleoptiles.
Pre-incubation with 1 mM $CuCl_2$ or $FeSO_4$ at pH 5.5, post-incubation in 30 mM ascorbate + 30 mM H_2O_2 ($CuCl_2$ pre-incubation) or 10 mM ascorbate + 10 mM H_2O_2 ($FeSO_4$ pre-incubation) at the pH indicated on the abscissa. Other experimental conditions as in Figure 3.

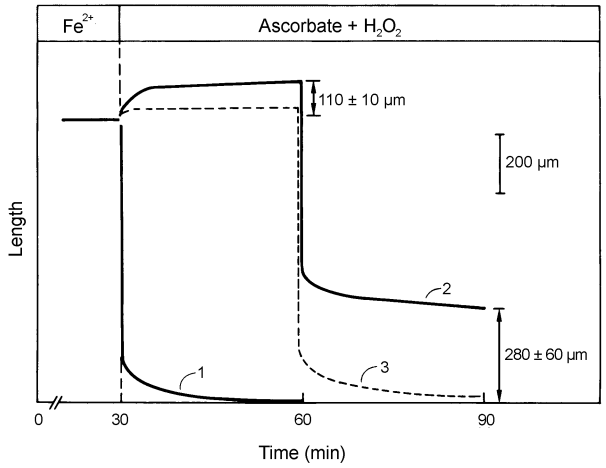

Figure 5. Demonstration of irreversible (plastic) extension during the OH-mediated creep reaction of isolated maize coleoptile cell walls.
Experimental conditions as in Figure 1, except that pre-incubation was in 1 mM $FeSO_4$ and post-incubation in 10 mM ascorbate + 10 mM H_2O_2 (pH 5.5). Curve 1: load removed before start of creep reaction induced by ascorbate + H_2O_2. Curve 2: load removed after 30 min creep reaction in the presence of ascorbate + H_2O_2. Curve 3: load removed after 30 min creep reaction in the absence of ascorbate + H_2O_2 (buffer control).

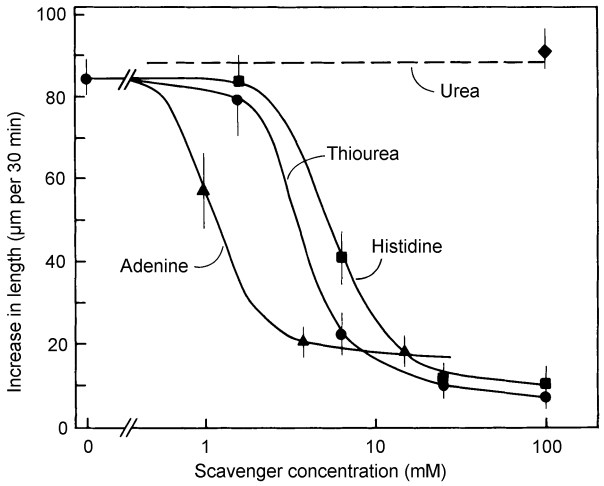

Figure 6. Inhibition of cell-wall extension by OH scavengers.
Experimental conditions as in Figure 1, except that adenine, histidine, thiourea or urea (used as a negative control) were included in the post-incubation medium together with ascorbate/H_2O_2. Extension was measured as increase in segment length during 30 min post-incubation in ascorbate/H_2O_2. Extension in buffer controls was subtracted.

not affect the creep reaction. Also, some potentially effective OH scavengers such as mannitol, ethanol, dimethylsulfoxide, tris and benzoate were unable to inhibit Cu-catalysed creep significantly, at least at concentrations up to 100 mM. The selective effectiveness of different OH scavengers has been reported previously (Gelvan *et al.*, 1992; Gutteridge, 1987), and can be explained by their unequal ability to compete with cell-wall targets for OH. Due to the strictly site-specific reactivity of OH, protection against molecular damage can be achieved only if the potential scavenger has sufficient metal-binding capacity to withdraw the catalytic metal ion from the target site. This is the case, for instance, for thiourea, but to a much lesser extent for mannitol (Halliwell and Gutteridge, 1989).

Axial organs of grasses and dicotyledons respond very similarly to added auxin with respect to the induction of elongation growth, despite striking qualitative differences in the polymer composition of their cell-wall matrix. Whereas typical primary walls of dicots contain xyloglucan as the major hemicellulose, and high amounts of pectin, the matrix of grass cell walls is mainly composed of xylan and mixed-linked glucan (Carpita and Gibeaut, 1993). Auxin brings about wall loosening in both types of wall in a remarkably similar fashion, although a common enzymatic or otherwise substrate-specific wall-loosening mechanism is difficult to imagine. On the other hand, because of their promiscuous reactivity towards biopolymers, it is to be expected that OH can cause wall loosening similarly in plants with very different types of primary wall. This prediction was investigated by comparing the creep behaviour of isolated walls from axial organs of different plant families. Figure 7 shows that walls from maize

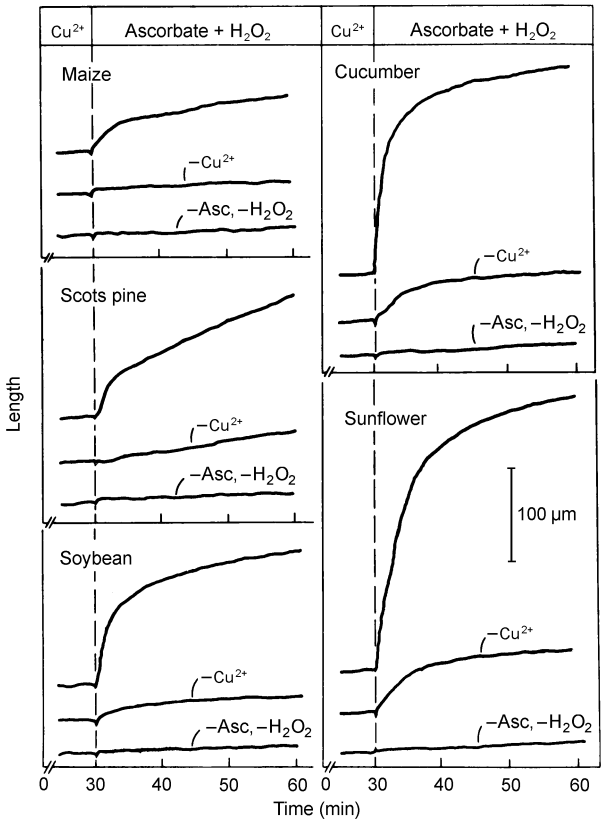

Figure 7. Induction of extension by generating OH in isolated cell walls of maize coleoptiles or hypocotyls of Scots pine, soybean, cucumber and sunflower seedlings.
Pre-incubation: 1 mM $CuCl_2$, post-incubation: 5 mM ascorbate + 5 mM H_2O_2 (pH 6.0). Wall specimens were stretched by a load of 40 g (maize), 20 g (Scots pine), 50 g (soybean), 8 g (cucumber) or 30 g (sunflower) selected so as to not exceed the breakage strength of the cell walls. Other experimental conditions as in Figure 1.

coleoptiles, as well as from cucumber, soybean, sunflower and Scots pine hypocotyls, demonstrate a qualitatively similar creep response to Cu-catalysed OH production, the extent of which varies depending on geometric tissue properties and applied force. Similar results were obtained if Cu^{2+} was replaced by Fe^{2+} during the pre-incubation period (data not shown). These results support the notion that the cell walls of growing plant organs are ubiquitously susceptible to OH with respect to the elicitation of creep, irrespective of their chemical composition.

Measuring creep by stretching isolated walls in an extensiometer can provide information on changes in the mechanical material properties of the walls which, however, do not necessarily reflect the chemorheological reactions governing wall loosening during cell growth *in vivo* (Cosgrove, 1993). It is therefore mandatory to test whether a putative wall-loosening factor invoked in controlling growth can also induce cell extension driven by turgor pressure in the living tissue. Figure 8 shows that OH generated from H_2O_2 and ascorbate in the presence of Cu ions in the cell walls of intact, turgid segments from maize coleoptiles or cucumber, soybean, sunflower and Scots pine hypocotyls induces a growth response that is comparable to the creep response obtained in the *in vitro* assay (Figure 7). Similar results were obtained if Cu^{2+} was replaced by Fe^{2+} during the pre-incubation period (data not shown).

The data presented so far provide evidence that OH, experimentally generated in the cell walls, is capable of inducing wall loosening and extension growth. This prompted the question whether this effect simulates the physiological wall-loosening reaction underlying auxin-induced growth, or represents a purely pharmacological phenomenon. To investigate this question, the effect of compounds acting as scavengers for O_2^-, H_2O_2 or OH on the growth response of maize coleoptiles was examined. Figure 9(a) shows that auxin-induced growth can be inhibited by micromolar concentrations of Mn-tetrakis-(methylpyridyl)porphin, an Mn–porphyrin complex catalysing the degradation of both O_2^- and H_2O_2 by acting as a substitute for superoxide dismutase and catalase (Day and Crapo, 1996; Day *et al.*, 1997). However, in contrast to the large enzyme proteins, this compound (molecular mass 909 Da) can easily penetrate into the cell-wall space. Under similar experimental conditions, the 'acid-growth' response induced in maize coleoptile segments by citrate buffer of pH 4.0 (Ding and Schopfer, 1997) was not affected by the O_2^-/H_2O_2 scavenger (Figure 9b). The involvement of OH in auxin-mediated growth was tested in similar experiments with the OH scavengers histidine, adenine and salicylate (Halliwell and Gutteridge, 1989). Thiourea could not be used in these *in vivo* experiments because this compound, similar to urea, is readily taken up into the cells and may lead to spurious results. Figure 10 shows that auxin-induced growth of maize coleoptile segments can be effectively inhibited by three physiologically compatible OH scavengers. None of these scavengers had an effect on acid-induced growth (data not shown). Taken together, these results demonstrate that OH, probably originating from O_2^- and H_2O_2 in a Haber–Weiss-type reaction in the cell wall, may be an essential component of the biochemical mechanism engaged in wall loosening during auxin-induced extension growth.

It has previously been shown that maize coleoptiles are capable of producing apoplastic O_2^-, and its dismutation product H_2O_2, in the growth-controlling outer epidermis (Frahry and Schopfer, 2001). If this is the source of O_2^- for the generation of OH involved in auxin-induced growth, it should be possible to stimulate O_2^- production in the epidermis by auxin. This prediction was tested using nitroblue tetrazolium chloride (NBT) as a histological probe for O_2^- (Frahry and Schopfer, 2001). Figure 11 shows that blue formazan product, resulting from the

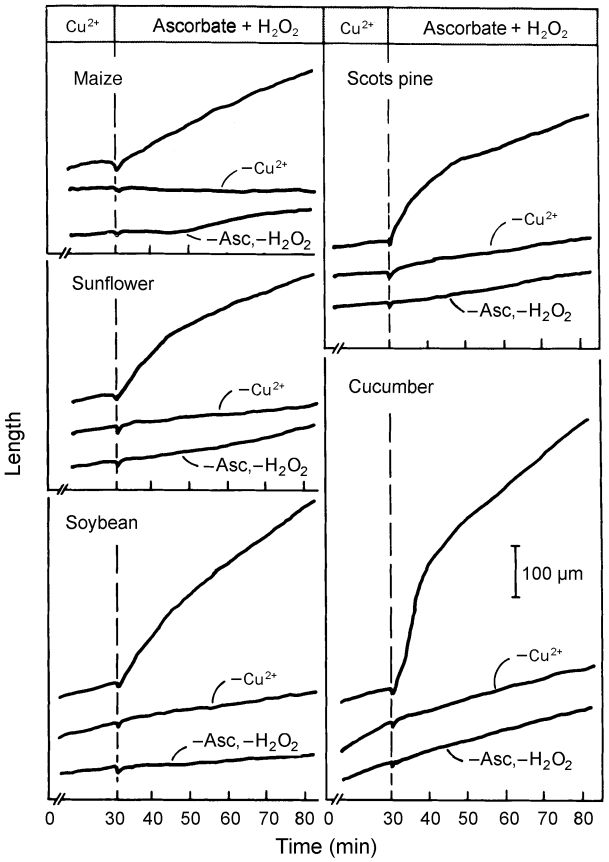

Figure 8. Induction of elongation growth by generating OH in intact segments of maize coleoptiles or hypocotyls of cucumber, soybean, sunflower and Scots pine seedlings.
Freshly harvested, abraded 1 cm segments were fixed in the growth recorder and kept for 1 h in 5 mM sodium succinate buffer (pH 6.0), followed by a pre-incubation of 30 min in the same buffer containing 1 mM $CuCl_2$ and a post-incubation in 5 mM ascorbate + 5 mM H_2O_2 in water (pH 6.0). The osmotic concentration of all solutions was 15 mosmol l^{-1}.

reduction of NBT by O_2^-, is specifically formed in the outer epidermis of the coleoptile. If coleoptile segments are incubated for 1 h in water for depleting endogenous auxin, the intensity of NBT staining is strongly reduced compared to freshly cut tissue. Application of auxin to the incubation medium restores the original staining intensity within 1 h. These experiments demonstrate that the production of O_2^-, and by inference, the subsequent generation of H_2O_2 and OH, is under the control of auxin.

Discussion

The biochemical mechanism of cell-wall loosening underlying elongation growth of plant organs represents an unsolved problem and is a matter of current debate (Cosgrove, 2000; Hoson, 1993). There is general agreement that this process involves the breakage of some kind of inter- or intramolecular tension-bearing bonds within the chemically heterogeneous composite material of primary cell walls. In such a complex system it is possible, and even likely, that several different factors contribute to the establishment of an extensible state of the wall, including agents that are involved in the turnover of polysaccharides or weaken the non-covalent associations between critical polymers. However, the decisive question is: Which one of these potential wall-loosening factors is actually rate-limiting, and thus *controlling*, growth on the background of necessary, but merely permissive factors in a particular situation? In the case of auxin-mediated elongation growth, physiological evidence imposes two important restrictions on the putative wall-loosening factor controlling wall extension:

1 Kinetic studies with organ segments demand that the wall-loosening agent causing elongation growth can be put into action within 10–20 min after the hormone has been recognized by competent cells, and loses activity within a similarly short period when the hormone is removed from the tissue (Bergfeld *et al.*, 1987; Ray and Ruesink, 1962). This *kinetic restriction* can be obeyed only by a wall-loosening agent with an effective lifetime of the order of minutes or less.

2 Auxin-induced growth of coleoptiles or hypocotyls, driven by the multidirectional force exerted by turgor pressure, is orientated almost exclusively in one direction, determined by the specific architecture of the cell walls. In elongating cells the cellulose microfibrils are typically orientated perpendicularly to the longitudinal axis, reinforcing the wall in girth similarly to the hoops of a barrel. This type of cell will expand preferentially in length, even though, for geometric reasons, the wall tension in girth is numerically twice as high as in length, theoretically favouring growth in girth over growth in length (Castle, 1937). It is the strong reinforcement by transverse cellulose microfibrils which counteracts this tendency and allows preferential cell expansion in length during the growth of axial plant organs (Carpita and Gibeaut, 1993). This *allometric restriction* can be obeyed only by a wall-loosening agent that attacks load-bearing bonds of interfibrillar matrix polymers but leaves the microfibril hoops and their anchorage in the matrix untouched, preventing their lateral slippage (Schopfer, 2000).

Considering the *kinetic restriction*, it is difficult to see, for instance, how a wall-degrading enzyme secreted into the cell wall under the influence of auxin could be put out of action within the period predicted by growth kinetics after cessation of auxin action. Theoretically, a wall-loosening mechanism based on a reversible activation of expansins by cell-wall acidification ('acid growth') would satisfy the kinetic constraints outlined above. However, there is evidence that auxin-induced growth is not accompanied by a drop in growth-efficient cell-wall pH sufficient for

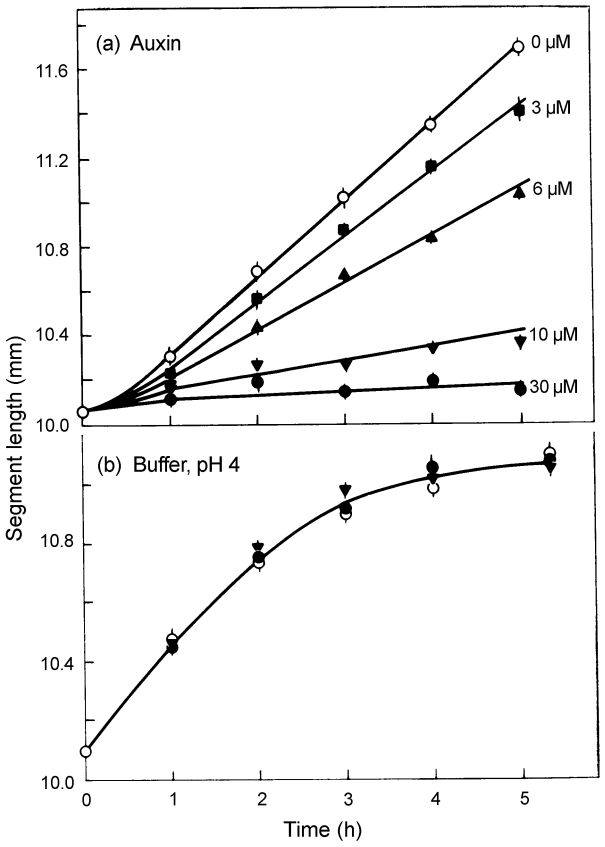

Figure 9. Inhibition of auxin-induced elongation growth by Mn-5,10,15,20-tetrakis(1-methyl-4-pyridyl)21*H*,23*H*-porphin, a scavenger of O_2^- and H_2O_2.
(a) Kinetics of elongation growth of abraded maize coleoptile segments incubated in water containing 20 µM indole-3-acetic acid and the indicated concentrations of the scavenger.
(b) Kinetics of acid-induced growth (10 mM sodium citrate buffer, pH 4.0) in the presence of the same scavenger concentrations.

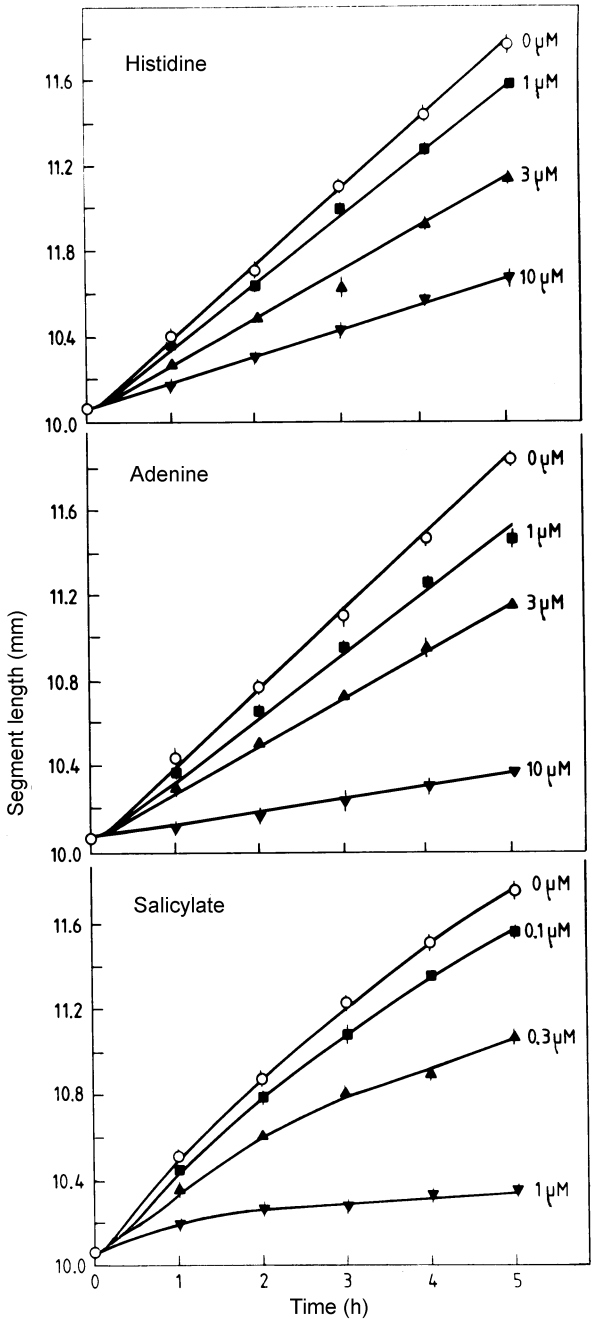

Figure 10. Inhibition of auxin-induced elongation growth by the OH scavengers histidine, adenine and salicylate.
Experimental details as in Figure 9(a).

inducing 'acid growth' in coleoptiles (Kutschera and Schopfer, 1985; Schopfer, 1993). In addition, in contrast to acid-induced wall loosening *in vitro*, acid-induced growth *in vivo* can be suppressed by metabolic inhibitors such as cyanide and azide (Ding and Schopfer, 1997). This contradicts the notion that the 'acid-growth' response can be simply explained by acid-induced activation of expansins in the wall. Moreover, if expansins act *in vivo* similar as proposed from experiments *in vitro* (Cosgrove, 2000; Whitney *et al.*, 2000) by disrupting hydrogen bonds between cellulose and hemicellulose at the surface of microfibrils, this would preferentially facilitate the lateral slippage of microfibrils relative to the cell-wall matrix. Hence, given the anisotropic distribution of tension and the hoop reinforcement of the cell wall, this mode of expansin action would cause growth in girth rather than growth in length, violating the allometric restriction dictated by wall architecture (Schopfer, 2000).

The experiments reported in the present paper support an alternative hypothesis proposing OH as the wall-loosening agent responsible for elongation growth. This hypothesis can be reconciled with the restrictions discussed above, and provides an explanation for a large body of older findings demonstrating a degradation of matrix polysaccharides during auxin-mediated growth (Bret-Harte and Talbott, 1993; Hoson, 1993; Talbott and

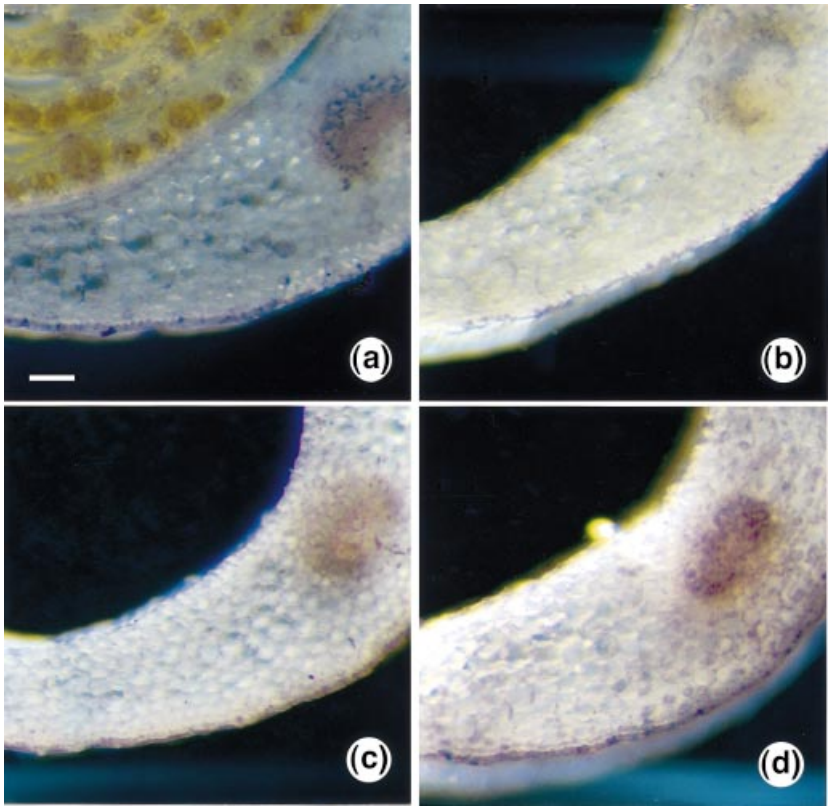

Figure 11. Effect of auxin on O_2^- production in the outer epidermis of maize coleoptiles. Hand-cut sections of living coleoptiles. Blue stain indicates reduction of nitroblue tetrazolium chloride to formazan by O_2^- (Frahry and Schopfer, 2001).
(a) Freshly cut tissue: the primary leaf (yellow) is still contained in the coleoptile. Coleoptile segments 1 cm long were incubated before preparation of sections for 1 h in water (b); 2 h in water (c); or 1 h in water + 1 h in water containing 20 μM indole-3-acetic acid (d). Bar = 100 μm.

Ray, 1992). OH represents a short-lived, highly reactive agent oxidatively cleaving polysaccharides at physiological pH *in vitro* (Fry, 1998; Schweikert *et al.*, 2000). The primary targets of OH may be matrix polysaccharides to which Cu or Fe ions (or peroxidase) can be ionically bound. Here it is shown that OH experimentally generated in isolated cell walls under tension causes a creep reaction phenomenologically very similar to the creep reaction induced by expansins in cucumber hypocotyl walls at pH 4.5 (McQueen-Mason *et al.*, 1992). Moreover, experimentally generated OH elicits a corresponding growth reaction in the living tissue.

The wall-loosening reaction demonstrated in these experiments appears to be equally effective in growing organs of gymnosperms, as well as monocotyledonous and dicotyledonous angiosperms. This illustrates the independence of OH-mediated wall loosening from species-specific differences in chemical wall properties, and suggests a generally applicable, new concept for the mechanism of wall loosening underlying extension growth. In the case of auxin-induced growth, the causal involvement of OH has been supported by the inhibitory action of scavengers directed against OH or its precursors O_2^- and H_2O_2. Moreover, it has been shown that auxin induces the formation of O_2^- in the growth-controlling epidermis of the coleoptile. It remains to be demonstrated that OH can be produced in the epidermal wall matrix *in vivo*, and that auxin promotes OH production when it induces growth. As a working hypothesis, it is proposed that OH can be generated from O_2^- and H_2O_2 by peroxidases (Chen and Schopfer, 1999) bound to the cell-wall matrix and, in this way, directs OH to the place where polymer cleavage induces cell-wall loosening.

Experimental procedures

Preparation of subapical segments (10 mm long, 3 mm below the tip) from 5-day-old etiolated maize (*Zea mays* L. cv. Perceval, from Asgrow, Bruchsal, Germany) coleoptiles was as described previously (Kutschera and Schopfer, 1985). Coleoptiles were abraded with polishing cloth before preparation of segments (Schopfer, 1993). Hypocotyl segments (10 mm long, 5 mm below the cotyledons) from 5-day-old etiolated seedlings of cucumber (*Cucumis sativus* L. cv. Bidretta GS, from J. Wagner GmbH, Heidelberg, Germany); sunflower (*Helianthus annus* L. cv. Gigantea, from U. Kutschera, Kassel, Germany); soybean (*Glycine max* L. cv. Recor R1 NT, from Rustica Sacon Pflanzenzucht GmbH, Dingelstedt, Germany); or 10-day-old etiolated seedlings of Scots pine (*Pinus sylvestris* L., from C. Appel, Samen und Pflanzen GmbH, Beerfelden, Germany) were prepared in the same way. Creep measurements were performed in a custom-built constant force extensiometer using frozen/thawed segments (Hohl and Schopfer, 1992; Kutschera and Schopfer, 1986). Growth measurements were performed in a custom-built growth recorder using intact segments (Kutschera and Schopfer,

1985). For testing the action of scavengers in long-term experiments, 10 1 cm coleoptile segments were incubated on a steel needle in 20 ml test solution and length changes were measured with a ruler. Mn-5,10,15,20-tetrakis(1-methyl-4-pyridyl)-21*H*,23*H*-porphin was obtained from Aldrich (Taufkirchen, Germany). The histochemical O_2^- assay was performed as described by Frahry and Schopfer (2001). Hand-cut sections (approximately 200 μm thick) were incubated for 40 min in 1 ml 10 mM sodium citrate buffer (pH 6.0) containing 0.5 mM nitroblue tetrazolium chloride (Serva, Heidelberg, Germany), 1 mM Na-diethyldithiocarbamate, 10 mM NaN_3, and 20 μM indole-3-acetic acid (if indicated). All experiments were conducted under normal laboratory light conditions at 25°C. Data are shown either as representative kinetics from single measurements or calculated means (± estimated SE) from at least four independent experiments.

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Note added in proof

In a paper that appeared after acceptance of the present contribution it was reported that cell-wall degradation in vitro by an unusual fungal endoglucanase can induce long-term wall extension (creep) and other changes in rheological wall properties similar to the changes observed after growth induction by auxin, but contrasting qualitatively with the wall-loosening action of expansins (Yuan et al., 2001). This observation further emphasises the profound mechanistic differences between wall-loosening reactions operating during auxin-induced growth or elicited by acid-activated expansins, respectively.

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