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ORIGINAL ARTICLE

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Evidence that hydroxyl radicals mediate auxin-induced extension growth

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Abstract Reactive oxygen intermediates, i.e. the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\cdot}OH$), are generally regarded as harmful products of oxygenic metabolism causing cell damage in plants, animals and microorganisms. However, oxygen radical chemistry may also play a useful role in polymer breakdown leading to wall loosening during extension growth of plant cells controlled by the phytohormone auxin. Backbone cleavage of cell wall polysaccharides can be accomplished in vitro by $^{\cdot}OH$ produced from H_2O_2 in a Fenton reaction or in a reaction catalyzed by peroxidase supplied with O_2 and NADH. Here, we show that coleoptile growth of maize seedlings is accompanied by the release of reactive oxygen intermediates in the cell wall. Auxin promotes release of $O_2^{\cdot-}$ and subsequent generation of $^{\cdot}OH$ when inducing elongation growth. Experimental generation of $^{\cdot}OH$ in the wall causes an increase in wall extensibility in vitro and replaces auxin in inducing growth. Auxin-induced growth can be inhibited by scavengers of $O_2^{\cdot-}$, H_2O_2 or $^{\cdot}OH$, or inhibitors interfering with the formation of these molecules in the cell wall. These results provide the experimental background for a novel hypothesis on the mechanism of plant cell growth in which $^{\cdot}OH$, produced from $O_2^{\cdot-}$ and H_2O_2 by cell wall peroxidase, acts as a wall-loosening agent.

Keywords Cell wall loosening · Coleoptile (maize) · Elongation growth · Hydroxyl radical · Superoxide radical · *Zea* (hydroxyl radical production)

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Abbreviations 2,4-D: 2,4-dichlorophenoxyacetate · DPI: diphenyleneiodonium · EPR: electron paramagnetic resonance · IAA: indole-3-acetate · 1-NAA: 1-naphthylacetate · POBN: α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone · SOD: superoxide dismutase · XTT: Na,3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate)

Introduction

Similar to mammalian leukocytes, plant cells are equipped with a plasma-membrane-bound redox enzyme system that can be activated, for example, in response to invading pathogens and catalyzes the extraprotoplasmic reduction of O_2 to $O_2^{\cdot-}$ at the expense of NAD(P)H (Wojtaszek 1997). As $O_2^{\cdot-}$ rapidly dismutates to H_2O_2, this "oxidative burst" results in the accumulation of H_2O_2 in the cell wall. $O_2^{\cdot-}$ and H_2O_2 are precursors for the production of $^{\cdot}OH$ catalyzed by iron or copper ions (Haber-Weiss reaction; Halliwell and Gutteridge 1989):

$$H_2O_2 + O_2^{\cdot-} \rightarrow {}^{\cdot}OH + OH^- + O_2.$$

This reaction can also be catalyzed enzymatically by peroxidases (Chen and Schopfer 1999). Thus, as peroxidases are generally present in plant cell walls in great abundance, $^{\cdot}OH$ can potentially be generated there whenever $O_2^{\cdot-}$ and H_2O_2 are available. Whereas $O_2^{\cdot-}$ and H_2O_2 are not exceptionally reactive, $^{\cdot}OH$ constitutes an extremely aggressive, short-lived oxidant disintegrating all kinds of organic molecules sufficiently close to its site of formation. For instance, rheumatoid arthritis in mammals can be traced back to the degradation of the polysaccharide hyaluronate by $^{\cdot}OH$ excessively produced in the lubrication fluid of joints (Hawkins and Davies 1996). Little is known about the generation and function of $^{\cdot}OH$ in plants, although there is evidence that $^{\cdot}OH$ can be produced during seed germination (Schopfer et al. 2001) and in defense reactions against pathogens (Kuchitsu et al. 1995; v. Tiedemann 1997). In

vitro, cell wall polysaccharides can be degraded by $^{\cdot}OH$ generated from H_2O_2 in the presence of ascorbate and Cu ions (Fry 1998) or enzymatically by peroxidase supplied with O_2 and a suitable reductant such as NADH (Chen and Schopfer 1999; Schweikert et al. 2000).

Cell extension during elongation growth of plant organs such as grass coleoptiles is mediated by an increase in the irreversible extensibility of growth-limiting cell walls under the control of the phytohormone auxin (Taiz 1984). It is generally accepted that auxin elicits the production of an as yet unknown wall-loosening factor mediating a chemorheological reaction that allows plastic deformation of the wall under the tensional force produced by turgor pressure. Although growth is generally accompanied by the synthesis of new wall material, wall loosening can only be brought about by bond-breaking reactions within or between existing wall polymers. Consequently, the present debate on the biochemical mechanism of cell extension growth concentrates on models in which either enzymatic polysaccharide cleavage (Hoson 1993) or weakening of hydrogen bonds between cellulose/hemicellulose molecules (Cosgrove 2000) is considered as a wall-loosening reaction. In fact, cell walls do contain several glucanases and transglycosylases cleaving wall polysaccharides in vitro, as well as expansins, i.e. proteins that induce expansion of isolated cell walls at acid pH by acting on non-covalent intermolecular bonds (Hoson 1993; Cosgrove 2000). However, no convincing evidence has been found so far that the reactions mediated by these proteins in vitro are involved in the wall-loosening reaction controlling auxin-induced growth. Envisaging the possibility that neither one of the previously proposed model reactions may lead to uncovering of the elusive wall-loosening factor, we explored the idea that $^{\cdot}OH$ can take over this function in vivo.

Materials and methods

Plant material and growth conditions

Seedlings of maize (*Zea mays* L. cv. Perceval; from Asgrow, Bruchsal, Germany) were grown on damp vermiculite for 4.5 days at 25.0 ± 0.3 °C in darkness interrupted after 3 days by a 10-min red light pulse (Frahry and Schopfer 2001). Coleoptiles were harvested and abraded with polishing cloth to remove the cuticular barrier to solutes (Schopfer 1993). Experiments were performed under normal laboratory light with intact coleoptiles or 1-cm coleoptile segments dissected 3 mm below the tip.

Demonstration of reactive-oxygen release

2′,7′-Dichlorofluorescin was prepared by hydrolyzing 2′,7′-dichlorofluorescin diacetate (Serva, Heidelberg, Germany) with pig liver esterase (Schopfer et al. 2001). The release of reactive oxygen intermediates by coleoptiles was visualized by embedding them for 10 min in agar medium (10 g l^{-1}) containing 50 μM dichlorofluorescin and 20 mM K-phosphate buffer (pH 6.0). Photographs were taken with an epifluorescence microscope (excitation 450–490 nm, emission 520 nm; Schopfer et al. 2001).

Determination of $O_2^{\cdot-}$ production

Batches of 10 abraded intact coleoptiles or 12 coleoptile segments were incubated in 3.5 ml 10 mM Na-citrate buffer (pH 6.0) containing 0.5 mM Na,3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT; Sutherland and Learmonth 1997). The rate of reduction of XTT by $O_2^{\cdot-}$ was measured photometrically for 15 min (Frahry and Schopfer 2001). Auxins and $O_2^{\cdot-}$-scavenging reagents were from Sigma (Deisenhofen, Germany) or Roche Molecular Biochemicals (Mannhein, Germany). Mn-desferrioxamine (green complex) was prepared from desferrioxamine mesylate (Sigma) and MnO_2 (Beyer and Fridovich 1989).

Determination of $^{\cdot}OH$ production

Batches of 20 coleoptile segments, cut into 1.2-mm sections, were incubated in 1.5 ml 20 mM suprapure K-phosphate buffer (pH 6.0) containing 850 mM ethanol/50 mM α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) as spin-trapping reagent (Ramos et al. 1992). Electron paramagnetic resonance (EPR) spectra of the hydroxyethyl-POBN adduct in the incubation medium were recorded at room temperature in a flat cell at 63 mW microwave power, 100 kHz modulation frequency, 0.2 mT modulation amplitude, 9.687 GHz microwave frequency, in a Bruker ESP X-band spectrometer. For the Fenton reaction, 50 μM Fe^{2+}-EDTA was mixed with 100 μM H_2O_2 in buffer and incubated for 30 min together with ethanol/POBN. Values of EPR signals were calculated from the maximum-signal/noise ratio of recorder traces and corrected, if necessary, by subtracting reagent blanks determined in parallel.

Determination of cell wall extensibility and elongation growth

Coleoptile segments killed by freezing/thawing were subjected to a stretching load of 40 g in a custom-built constant-force extensiometer (Ding and Schopfer 1997). After a pre-incubation with 1 mM $FeSO_4$ in 50 mM Na-succinate buffer (pH 6.0) the solution was replaced by 10 mM H_2O_2 + 10 mM Na-ascorbate in the same buffer. For measuring the effect of $^{\cdot}OH$ on elongation growth, living segments were subjected to a pre-incubation with 1 mM $FeSO_4$ in 1 mM Na-succinate buffer (pH 6.0) followed by 1 mM H_2O_2 + 1 mM Na-ascorbate (adjusted to pH 6.0) in a custom-built growth recorder (Schopfer 1993). For measuring the effect of inhibitors on auxin-mediated growth, batches of 10 segments were lined up on a syringe needle and elongation was determined with a ruler. The pH was adjusted to 6.0. Diphenyleneiodonium (DPI; dissolved in dimethyl sulfoxide) was from Biomol (Hamburg, Germany); other inhibitors were from Sigma.

Results

Production of reactive oxygen intermediates by coleoptiles

In contrast to the growth response elicited experimentally by acid pH, auxin-mediated growth critically depends on the presence of O_2 (Ray and Ruesink 1962; Hager et al. 1971). This observation has been interpreted to mean that only the latter depends on respiration, but is also consistent with a more specific role of O_2 in the wall-loosening reaction. Using the oxidation of dichlorofluorescin to fluorescent dichlorofluorescein, an in vivo assay for the "oxidative burst" in leukocytes (Bass et al. 1983), it can be shown that intact growing coleoptiles release reactive oxygen intermediates into the

surrounding medium in an O_2-dependent reaction if the diffusion barrier of the cuticle is removed by abrasion (Fig. 1). This assay detects production of reactive oxygen intermediates mainly at the level of H_2O_2 (Bass et al. 1983), originating from the dismutation of $O_2^{\cdot-}$ formed in the epidermis of the coleoptile (Frahry and Schopfer 2001).

Effect of auxin on the production of superoxide

The apoplastic production of $O_2^{\cdot-}$ by plant tissues in vivo can be specifically determined with a tetrazolium-based assay using XTT as a sensitive probe (Frahry and Schopfer 2001; Sutherland and Learmonth 1997). Using this assay, Fig. 2 shows that the rate of $O_2^{\cdot-}$ production by coleoptiles incubated in buffer decreases concomitantly with the decrease in growth rate accompanying the depletion of endogenous auxin (indole-3-acetate, IAA). Reconstitution of growth by adding auxin to the incubation medium increases the rate of $O_2^{\cdot-}$ production in a reversible manner. The synthetic auxins 2,4-dichlorophenoxyacetate (2,4-D) and 1-naphthylacetate (1-NAA), but not the inactive analogs 2,3-D and 2-NAA, replaced IAA in inducing $O_2^{\cdot-}$ production (Table 1, coleoptile segments). The assay reaction could only partly be inhibited by superoxide dismutase (SOD), presumably because of limited penetration of the enzyme into the cell wall (Frahry and Schopfer 2001). The low-molecular-weight SOD mimic Mn-desferrioxamine (Beyer and Fridovich 1989) removed $O_2^{\cdot-}$ almost completely, demonstrating the specificity of the assay reaction for $O_2^{\cdot-}$ (Table 1, intact coleoptiles). Production of $O_2^{\cdot-}$ by coleoptiles was drastically increased by external NADH, but not by NADPH, in the absence and presence of IAA, in agreement with the idea that a plasma-membrane-bound NADH oxidase accepting NADH

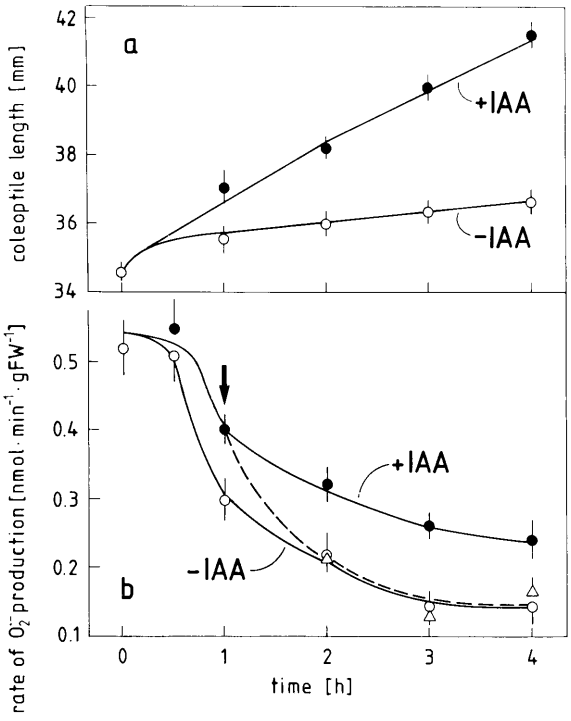

Fig. 2a, b Effect of auxin on growth (**a**) and $O_2^{\cdot-}$ release (**b**) of maize coleoptiles. Abraded coleoptiles were incubated for 1 h in water followed by up to 4 h in Na-citrate buffer (10 μM, pH 6.0) without (*–IAA*) or with (*+IAA*) 20 μM IAA. $O_2^{\cdot-}$ release was determined with a tetrazolium-based assay. Reversibility of IAA action on $O_2^{\cdot-}$ release was tested by removing IAA after 1 h (*arrow*). Means of four to six experiments ± SE

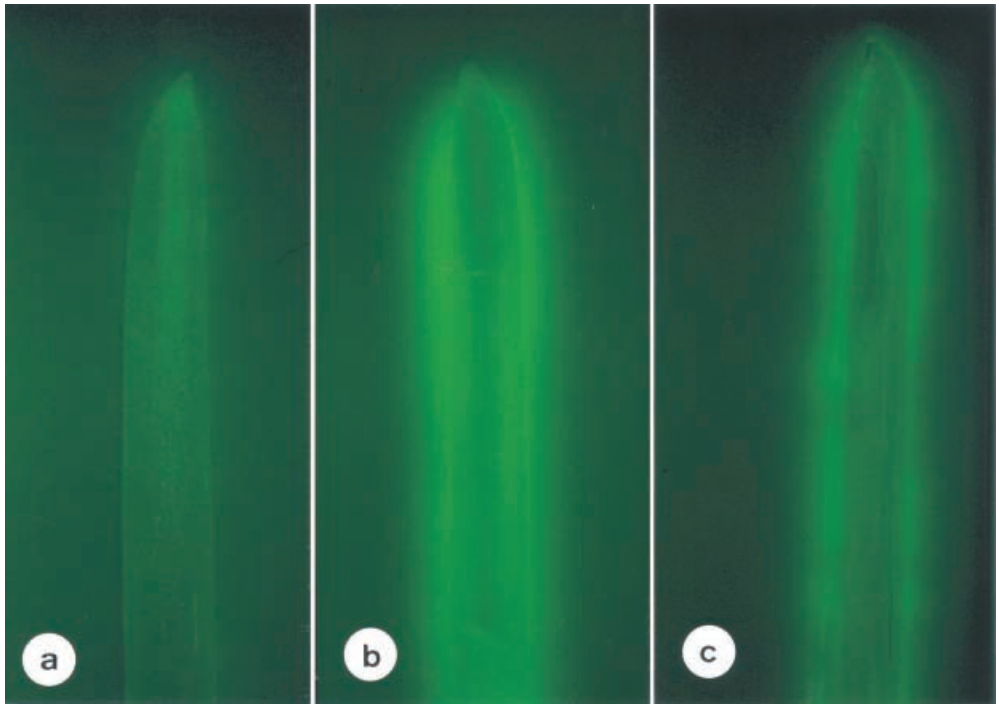

Fig. 1a–c Release of reactive oxygen intermediates by maize (*Zea mays*) coleoptiles. Non-abraded (**a**) or abraded (**b, c**) coleoptiles were embedded in agar medium, containing dichlorofluorescin as a non-permeant probe converted to fluorescent dichlorofluorescein by reactive oxygen intermediates, in air (**a, b**) or flushed with N_2 (**c**)

Table 1 Effect of active auxins (IAA, 2,4-D, 1-NAA) and inactive auxin analogs (2,3-D, 2-NAA), $O_2^{\cdot-}$ scavengers (SOD, Mn-desferrioxamine) and putative electron donors (NADH, NADPH) on $O_2^{\cdot-}$ production by maize coleoptiles. Abraded 1-cm coleoptile segments were incubated for 1 h in water followed by 1 h in Na-citrate buffer containing auxins or inactive auxin analogs (20 μM). Abraded intact coleoptiles were incubated similarly except that scavengers and electron donors were added in the absence or presence of IAA (20 μM). $O_2^{\cdot-}$ production was measured in the presence of the respective compounds as in Fig. 2. Means of four to six experiments ± SE

Treatment	Rate of $O_2^{\cdot-}$ production [nmol min^{-1} g FW^{-1}]	
	–IAA	+IAA
Coleoptile segments		
Control	0.18 ± 0.02	0.24 ± 0.02
+2,4-D	0.23 ± 0.01	–
+2,3-D	0.17 ± 0.02	–
+1-NAA	0.25 ± 0.03	–
+2-NAA	0.19 ± 0.02	–
Intact coleoptiles		
Control	0.30 ± 0.03	0.40 ± 0.02
+ SOD (50 μg ml^{-1})	0.20 ± 0.03	0.25 ± 0.03
+ Mn-desferrioxamine (0.5 mM)	0.05 ± 0.02	0.04 ± 0.01
+ NADH (200 μM)	1.21 ± 0.16	2.11 ± 0.22
+ NADPH (200 μM)	0.24 ± 0.01	0.24 ± 0.02

from the apoplastic space (Lin 1982; Frahry and Schopfer 2001) is responsible for the reaction (Table 1, intact coleoptiles).

Effect of auxin on the production of hydroxyl radicals

It has previously been shown that $O_2^{\cdot-}$ released by the epidermis of maize coleoptiles can be converted to H_2O_2 and that the epidermal walls contain peroxidase (Frahry and Schopfer 2001), an enzyme capable of generating $^{\cdot}OH$ in the presence of $O_2^{\cdot-}$ and H_2O_2 (Chen and Schopfer 1999). The detection of $^{\cdot}OH$ production by living plant cells is technically difficult and has so far not been reported except in germinating seeds (Schopfer et al. 2001) and host-pathogen relationships (Kuchitsu et al. 1995; v. Tiedemann 1997). We tested $^{\cdot}OH$ production in maize coleoptiles in vivo by EPR spectroscopy using ethanol/POBN as a spin-trapping reagent (Ramos et al. 1992). Figure 3a shows that the EPR spectrum of the hydroxyethyl-POBN radical adduct formed in the presence of coleoptile tissue (trace 1) resembles the EPR spectrum obtained with an $^{\cdot}OH$-generating Fenton reaction system (trace 4). The signal can be suppressed by scavenging $^{\cdot}OH$ with thiourea (trace 2) or inactivating enzyme activities with trichloroacetate (trace 3). Using the signal size of EPR spectra measured under standardized conditions as a quantitative index for $^{\cdot}OH$, Fig. 3b shows that a pretreatment with the synthetic auxin 2,4-D induces an increased level of $^{\cdot}OH$ in the incubation medium of coleoptile tissue whereas the inactive analog 2,3-D is ineffective. The production of $^{\cdot}OH$ could be detected by spin-trapping over at least

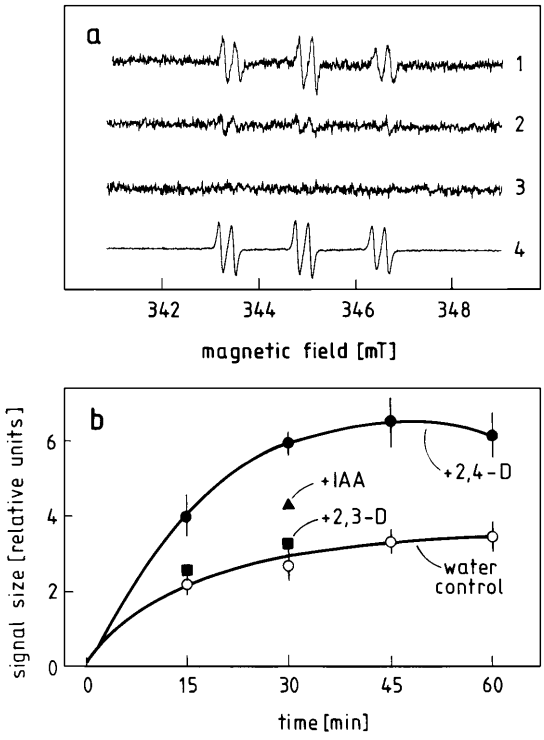

Fig. 3a, b Effect of auxin on $^{\cdot}OH$ production by maize coleoptiles. **a** EPR spectra of hydroxyethyl-POBN adduct diagnostic for $^{\cdot}OH$ formed by coleoptile tissue. *Trace 1* Coleoptile segments were incubated for 1 h in water followed by 1 h in 20 μM 2,4-D. $^{\cdot}OH$ production was subsequently assayed by incubating the tissue for 30 min in 20 μM 2,4-D together with ethanol/POBN used as spin-trapping reagent. *Trace 2* As in *1*, but 1 mM thiourea added together with 2,4-D. *Trace 3* As in *1*, but tissue killed by incubating it for 10 min in 10% trichloroacetate followed by washing with buffer before adding ethanol/POBN. *Trace 4* Reference spectrum determined with the Fenton reaction under similar conditions. **b** Signal size of EPR spectra measured as in **a**, *trace 1*, in the presence and absence of 2,4-D, as a function of incubation time in ethanol/POBN. Data obtained with the biologically inactive auxin analog 2,3-D (20 μM) or the natural growth hormone IAA (20 μM) are included at 15 and 30 min. Means of four experiments ± SE

60 min, in agreement with the relative long half-life of the hydroxyethyl-POBN adduct (Ramos et al. 1992). At equimolar concentration the natural auxin IAA was less active than 2,4-D, presumably due to inactivation by tissue peroxidases at the high tissue/medium ratio necessary in these experiments. Peroxidase inhibitors (KCN and NaN_3; Chen and Schopfer 1999) or scavengers of $O_2^{\cdot-}$ ($CuCl_2$ and SOD; Zancany et al. 1995), H_2O_2 (KI; Halliwell and Gutteridge 1989) and $^{\cdot}OH$ (desferrioxamine and thiourea; Halliwell and Gutteridge 1989) reduced the level of $^{\cdot}OH$ detected by EPR (Table 2).

Effect of hydroxyl radicals on cell wall extension in vitro and in vivo

Having established that coleoptiles produce $O_2^{\cdot-}$ giving rise to the formation of $^{\cdot}OH$ in an auxin-inducible

Table 2 Effect of inhibitors of peroxidase (KCN, NaN_3), NAD(P)H oxidase ($ZnCl_2$) and scavengers of $O_2^{\cdot-}$ ($CuCl_2$, SOD), H_2O_2 (KI) or $^{\cdot}OH$ (desferrioxamine, thiourea) on $^{\cdot}OH$ production by maize coleoptile segments. $^{\cdot}OH$ production was determined from EPR spectra obtained after applying the inhibitors together with 2,4-D as in Fig. 3a, trace 2. Urea was included as a control substance without scavenger activity (Halliwell and Gutteridge 1989)

Treatment	Relative amount of $^{\cdot}OH$ (%)
Control	100 ± 3
+ KCN (1 mM)	15 ± 5
+ NaN_3 (5 mM)	44 ± 2
+ $ZnCl_2$ (5 mM)	22 ± 8
+ $CuCl_2$ (0.5 mM)	< 10
+ SOD (50 μg ml^{-1})	55 ± 8
+ KI (10 mM)	36 ± 7
+ Desferrioxamine (10 mM)	28 ± 10
+ Thiourea (1 mM)	24 ± 10
+ Urea (100 mM)	81 ± 7

manner, we tested whether the experimental generation of $^{\cdot}OH$ in the cell wall can cause wall loosening and growth. Figure 4a shows that isolated cell walls impregnated with Fe^{2+} ions can be induced to extend under load by treating them with H_2O_2 + ascorbate, a conventional reaction system producing $^{\cdot}OH$ (Halliwell and Gutteridge 1989). Scavengers of $^{\cdot}OH$ such as thiourea inhibit the reaction, confirming that $^{\cdot}OH$ is the effective wall-loosening agent (data not shown). The same $^{\cdot}OH$-generating reaction can be used for inducing elongation growth in living coleoptile segments (Fig. 4b). The initial growth rate induced by $^{\cdot}OH$ in this experiment is similar to the growth rate that can be induced by a saturating concentration of auxin. The subsequent decrease in extension observed both in vitro and in vivo is presumably due to the decreasing rate of $^{\cdot}OH$ production because of substrate exhaustion. This is indicated by the observation that extension can be reinduced by fresh incubation medium (data not shown).

Basically similar results were obtained in experiments in which Fe^{2+} was replaced by Cu^{2+} in the presence of H_2O_2 + ascorbate for catalyzing the production of $^{\cdot}OH$ in the cell walls, or by inducing the endogenous wall peroxidase to produce $^{\cdot}OH$ in the presence of H_2O_2 + NADH (data not shown).

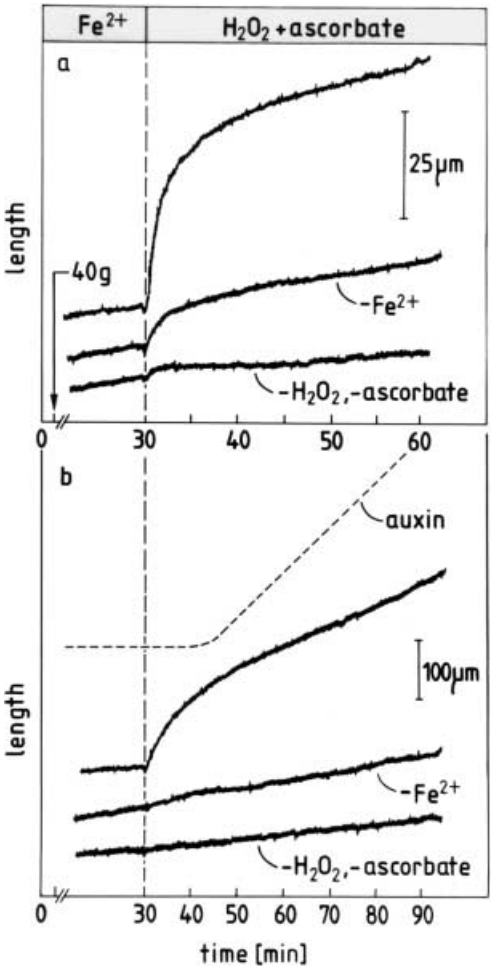

Fig. 4a, b Induction of cell wall extension in vitro (**a**) and elongation growth (**b**) by generating $^{\cdot}OH$ in the walls of maize coleoptile segments. **a** Wall extension was measured by stretching abraded, killed segments in an extensiometer. After impregnation with $FeSO_4$, segments were incubated in H_2O_2 + ascorbate to initiate $^{\cdot}OH$ production in the cell walls. Controls were run by omitting either $FeSO_4$ or H_2O_2 + ascorbate from the incubation buffers. **b** Elongation growth was measured by subjecting abraded, living segments in a growth recorder to a similar incubation protocol. Typical traces out of six experiments. The time course of elongation induced by IAA (20 μM) is included for comparison

Effect of inhibitors of reactive-oxygen production on auxin-mediated growth

If auxin-dependent elongation growth is mediated by peroxidase-catalyzed $^{\cdot}OH$ production in the cell wall, it should be sensitive to inhibition by agents interfering with $O_2^{\cdot-}$ formation and the reactions involved in the generation of $^{\cdot}OH$ from $O_2^{\cdot-}$ and H_2O_2. This prediction was tested by employing various peroxidase and NAD(P)H oxidase inhibitors or $O_2^{\cdot-}$ and $^{\cdot}OH$ scavengers. As representative examples, Fig. 5 shows the effect of the $^{\cdot}OH$ scavenger benzoate (Halliwell and Gutteridge 1989) and the NAD(P)H oxidase inhibitor diphenyleneiodonium (DPI; Cross and Jones 1986) on the time course of auxin-induced elongation growth of maize coleoptile segments. Table 3 summarizes the results of corresponding experiments with a number of additional inhibitors known to interfere with peroxidase activity (Halliwell and Gutteridge 1989; Chen and Schopfer 1999) or NAD(P)H oxidase activity (Cakmak 2000), or to act as $O_2^{\cdot-}$ or $^{\cdot}OH$ scavengers (Beyer and Fridovich 1989; Halliwell and Gutteridge 1989; Zancany et al. 1995; Cakmak 2000). The effect of these substances on the acid-induced growth response of coleoptile segments (Ding and Schopfer 1997) was determined in parallel experiments in order to test for unspecific growth inhibition (Table 3). These data show that auxin-induced growth can be suppressed by compounds inhibiting the

formation of ·OH and its precursors under conditions where acid-induced growth is not affected. Since adenine and histidine have only a weak buffering capacity at pH 6 and do not influence the acid-induced growth, pH and osmotic effects can be excluded. Moreover, there is indirect evidence for the involvement of NADH oxidase and peroxidase in the production of the wall-loosening agent involved in growth. In agreement with these results is the observation that general antioxidants such as reduced glutathione or dithiothreitol are effective inhibitors of auxin-induced growth (Morré et al. 1995 and own unpublished results).

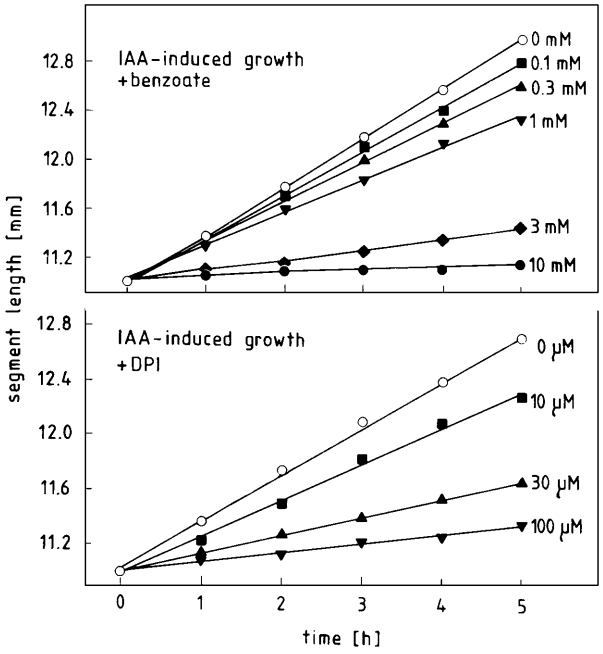

Fig. 5 Inhibition of auxin-induced growth of maize coleoptile segments by the ·OH scavenger benzoate and the NAD(P)H-oxidase inhibitor DPI. Abraded coleoptile segments were incubated for 1 h in water and then in 20 µM IAA + indicated concentrations of Na-benzoate or DPI (plus < 14 mM dimethyl sulfoxide, ineffective in controls) at pH 6.0. Means of four experiments

Discussion

Taken together, our experiments provide verification for the hypothesis that ·OH is causally involved in the chemorheological wall-loosening reaction responsible for auxin-controlled growth of maize coleoptiles. This conclusion can be based on four lines of evidence:

i. ·OH, as well as its precursor $O_2^{\cdot-}$, can be produced by coleoptiles, and exogenous auxin promotes the release of these reactive oxygen intermediates into the cell wall.
ii. In vitro, ·OH, generated at the expense of $O_2^{\cdot-}$ and its dismutation product H_2O_2 in a peroxidase-catalyzed reaction, causes backbone cleavage of cell wall polysaccharides (Schweikert et al. 2000).
iii. The experimental generation of ·OH in the cell wall causes wall loosening and short-term extension growth similarly to auxin.
iv. Auxin-mediated elongation growth can be suppressed by inhibiting the generation of ·OH in the wall.

The apoplastic production of $O_2^{\cdot-}$ can be attributed to a cyanide-insensitive, NADH-dependent O_2^{cdot-} synthase activity localized in the plasma membrane which has

Table 3 Effect of inhibitors of peroxidase and NAD(P)H oxidase, and scavengers of $O_2^{\cdot-}$ or ·OH on auxin-induced (20 µM IAA) and acid-induced (10 mM Na-citrate buffer, pH 4.0) growth of maize coleoptile segments. Relative initial growth rates were calculated from time-course measurements as shown in Fig. 5

Treatment	Extension rate (%)	
	Auxin-induced	Acid-induced
Control	100 ± 8	100 ± 10
+ *Peroxidase inhibitors*:[a]		
KCN (1 mM)	20 ± 5	–
NaN_3 (0.3 mM)	6 ± 2	–
Na_2S (10 mM)	5 ± 2	78 ± 13
Hydroxylamine (3 mM)	5 ± 2	113 ± 19
1,10-Phenanthroline (3 mM)	11 ± 4	147 ± 12
2, 2′-Bipyridyl (10 mM)	9 ± 2	91 ± 17
+ *NAD(P)H oxidase inhibitors*:		
DPI (0.1 mM)[b]	25 ± 5	100 ± 8
$ZnCl_2$ (0.3 mM)	11 ± 2	102 ± 2
+ $O_2^{\cdot-}$ *scavengers*:		
Mn-desferrioxamine (3 mM)	21 ± 2	99 ± 8
$CuCl_2$ (0.03 mM)	8 ± 2	94 ± 16
Tiron (1 mM)	36 ± 5	100 ± 10
+ ·OH *scavengers*:		
Adenine (10 mM)	21 ± 3	69 ± 4
Histidine (10 mM)	37 ± 2	114 ± 4
Na-benzoate (3 mM)	39 ± 5	78 ± 9
Tris (3 mM)	35 ± 3	89 ± 11
Na-salicylate (3 mM)	15 ± 4	65 ± 22

[a]Can also act as ·OH scavengers
[b]Also inhibits peroxidase (Frahry and Schopfer 1998)

been characterized previously (Frahry and Schopfer 2001). This enzyme activity is reminiscent of the inducible NAD(P)H oxidase supposed to mediate the "oxidative burst" induced by pathogen-derived elicitors in plants and animals (Wojtaszek 1997). NADH-dependent redox activity of plasma membranes has been reported previously by numerous investigators, although the natural reduction product as well as the physiological function of this enzyme system has remained elusive (for reviews, see Lüthje et al. 1997; Bérczi and Møller 2000). It is interesting to note in this context the work by Morré and collaborators who described an NADH-consuming enzyme activity in the plasma membrane of soybean hypocotyls, which could be stimulated by auxin in vitro (Morré et al. 1988) or in vivo (Hicks and Morré 1998) and was assumed to have a function in growth regulation by acting as a protein disulfide reductase (Morré et al. 1995; Morré 1998). The relationship, if any, between this enzyme activity and the $O_2^{\cdot-}$-producing NADH oxidase activity described in the present report remains to be clarified.

In the maize coleoptile, the auxin-inducible production of $O_2^{\cdot-}$ and derived reactive oxygen intermediates is largely confined to the outer epidermis (Frahry and Schopfer 2001). It has previously been shown that the epidermis represents the target tissue for the action of auxin on elongation growth and related phenomena, for instance the appearance of "osmiophilic particles" at the plasma membrane/cell-wall interface (Kutschera et al. 1987). The biochemical properties and the functional significance of these particles have not yet been clearly elucidated. In the light of the present results it appears possible that these structures result from the cross-linking action of reactive oxygen intermediates on secreted cell-wall proteins (Schindler et al. 1994). Interestingly, strikingly similar osmiophilic deposits at the plasma membrane/cell-wall interphase are formed when cross-linking of glycoproteins in the presence of peroxidase and H_2O_2 is induced in mesophyll cells as a defense response to invading bacteria (Brown et al. 1998).

Based on the results of this and previous studies (Chen and Schopfer 1999; Schweikert et al. 2000; Frahry and Schopfer 2001) we propose that auxin promotes the apoplastic production of $O_2^{\cdot-}$ by a plasma-membrane-bound NADH oxidase, followed by the conversion of part of the $O_2^{\cdot-}$ to H_2O_2. These reactive oxygen intermediates can be utilized as substrates by peroxidase bound to cell wall polymers for generating $^{\cdot}OH$ close to load-bearing sites in the polysaccharide wall matrix. Site-specific (Halliwell and Gutteridge 1989) polymer cleavage by ^{c}dotOH would then cause a controlled mechanical weakening of the wall structure and relaxation of wall tension resulting in irreversible cell extension in the presence of turgor pressure. This aspect is further elaborated in a forthcoming paper (Schopfer, in press). There is evidence that these processes are initiated in the growth-limiting outer epidermal wall of the coleoptile (Frahry and Schopfer 2001). This hypothesis, at present based mainly on qualitative experimental evidence, is now open for critical testing at the quantitative level.

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Note added in proof. In a recent paper, Joo et al. report on a transient auxin-dependent production of reactive oxygen intermediates in gravistimulated maize roots and isolated root protoplasts [Joo JH, Bae YS, Lee JS (2001) Plant Physiol 126:1055–1060]. Based on these and additional results the authors suggest a role for reactive oxygen intermediates in gravitropic signal transduction.